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Quantitation and Identification of Free Sugars from Vascular Plants by Gas-liquid Chromatography with Mass Spectrometry

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QUANTITATION AND IDENTIFICATION OF FREE SUGARS FROM VASCULAR PLANTS BY GAS-LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements of the degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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To my wife, Kathryn,

our daughter, Kristina,

and our son, Daniel

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INTRODUCTION

The objectives of this thesis are both the further development of a method for the analysis of free sugars in plant tissues, and also the application of this method to tissues from vascular plants. The applied methodology is often referred to as the PAAN and/or PAKO procedure, an acronym for peracetylated aldononitrile and /or peracetylated ketooxime derivatives. The two step derivatization procedure (oxime formation followed by peracetylation)will also convert polyols and non-reducing sugars to their corresponding peracetylated compounds. During the past decade there has been extensive application of this procedure, and also development of various aspects of the approach. A recent Master's thesis from SDSU, that of Stacy L. Unruh (1) investigated both conditions for free sugars in plant tissues, and also reviewed (relative to 1983) the literature in this area. The following literature review was completed during the summer of 1986; it is based on Unruh's literature search, and emphasizes developments during the 1981-1986 period. Well defined conditions for the effective quantitation of the principle free sugars (D-glucose, sorbitol, D-fructose, and sucrose) found in plant tissues were a major contribution of Unruh's study. (To simplify the

following discussion, the polyol, sorbitol is referred to as a "sugar").

The developmental objectives of this study are as follows. Firstly, to further investigate the methodology of Unruh for the analyses of sugars. Secondly, to employ digital intergration referenced to an internal standard for quantitation. Thirdly, to precisely establish the detector responses for the major free sugars present in plant tissues. Fourthly, to extend the PAAN/PAKO method (in terms of gas chromatographic, GC, and mass spectral, MS, analyses) to complex glycosides and disaccharides. The application objective has been to use the PAAN/PAKO procedures to analyze tissue from vascular plants, especially material from stems and leaves -- material which had resisted the procedure developed by Unruh. Preliminary reports of these studies have been presented at the Midwest Regional meetings of the American Chemical Society in 1984 at Springfield, Mo. (2), and in 1985 at Carbondale, Il. (3, 4).

Early developments of the PAAN/PAKO method have been discussed by Unruh (1). Important PAAN/PAKO papers include, the first GC application by Lance and Jones (5), the first MS application by Dmitriev et al. (6), and the extension of the GC/MS procedure to both aldoses and ketoses by Seymour et al. (7,8). The PAAN/PAKO procedure

has two major areas of application; the first area being the analysis of free non-O-methylated sugars -- which is of central interest in this thesis. The second area of application is the analysis of partially O-methylated saccharides which arise in the hydrosylates from per-methylation studies for the structural determination of polysaccharides. However, this second area is of interest to the study herein with regard to advances in procedures for the derivatization technique. Importantly, it should be noted that in the following literature survey reference is not made to: (a) the combined PAAN/PAKO procedure to simultaneously survey ketoses and aldoses, and (b) the use of the PAAN/PAKO procedure for the direct analysis of any free sugars from plant tissue. In addition, only limited references have been found to the use of an internal standard and/or for area integration for purposes of quantitation of sugars -- for these derivatives or for other systems.

The use of PAAN derivatives for identification and/or quantitation of non-O-methylated saccharides.

The PAAN procedure has been employed for analysis of sugar composition of compounds isolated from plants, for example, Becchi et al. (9) first isolated a new

saponin from Chrysanthellum procumbus, hydrolyzed this compound, and finally used the PAAN procedure to identify D-glucose, D-xylose, and L-rhamnose as the carbohydrate components. Belcher et al. (10) analyzed the total sugar content of the "gossypol" glands in cotton anthers (after hydrolysis) with the PAAN procedure, and Nicollier et al. (11, 12) employed the PAAN procedure to establish the component sugars (glucose and rhamnose) in two new flavinoids which had been extracted from the flowers of white clover (Melilotus alba). Schultz et al. (13) used the PAAN method to monitor sugars arising from enzymatic hydrolysis of steam explosion products of hemicelluloses from a variety of sources (hard wood, rice hulls, etc.). Hedin et al. (14) employed the PAAN procedure to examine the chemical composition of whorl tissue from strains of corn (Zea mays L.) which were either resistant or non-resistant to the southwestern corn borer. In a similar manner, Maningat and Juliano (15) used the PAAN procedure to establish the component sugars in the cell walls of rice bran. In all the above studies, non-O-methylated sugars from plant sources were analyzed, but all of these sugars arose from hydrosylates of larger molecules which had been separated from the plant tissue -- not from free sugars, in the sense of extracting components from plant tissue without the breaking of covalent bonds. Although

Maningat and Juliano (15) extensively employed the PAAN procedure for analysis of sugars from hydrosylates, in the same article they employed wet-chemical means to analyze for the free sugar content of the original tissues. Both Whitfield *et al.* (16) and Pringle *et al.* (17) used the PAAN method for analysis of the composition of exopolysaccharides and lipopolysaccharides from microbial strains (respectively, *Santhomonas campestris* and *Pseudomonas fluorescens*). Gage *et al.* (18) made use of a combination of P-31 Nuclear Magnetic Resonance (NMR) and the PAAN procedure to identify a phosphomannan in the cell wall of the yeast, *Saccharomyces cerevisiae*. In all cases these sugar analyses were performed on hydrosylates of polymers which had been extracted from the natural source. In addition to the above reports, the PAAN procedure has been applied to sugars which arise from conditions (and contaminated with impurities) that are profoundly different from those involved with the extracts of natural products. Nyhammar *et al.* (19) used PAAN derivatives to study the products which arose in the Maillard reaction through the Strecker degradation. Shafizadeh and co-workers routinely employed the PAAN derivatization procedure to examine products arising from the pyrolysis of polysaccharides (20) and wood (21, 22).

In addition to the above relatively conventional sugar analyses, there are several reports which employ the PAAN method in conjunction with GC-MS for identifying new saccharides (in each case as residues which were originally incorporated into polymers) from natural sources-- an aspect of the PAAN procedure which has great potential. Jackson *et al.* (23, 24) reported finding the 3-O-methyl-L-rhamnose residue in hydrosylates of extracellular and capsular polymers from microbial strains of *Rhizobium*. This naturally occurring O-methylated sugar is independent of the below discussed O-methyl saccharide derivatives, which are products of the synthetic permethylation process. Similarly, Mort and Bauer (25, 26) identified the 4-O-methyl-galactose residue in extracellular and capsular polysaccharides of *Rhizobium japonicum*. Romanowska *et al.* (27) identified 4-deoxy-D-arabinohexopyranosyl residues in a lipopolysaccharide isolated from a strain of Citrobacter, and concluded that the original compound was a homopolymer. Edwards *et al.* (28) identified intracellular mannitol as a product of the metabolism of D-glucose in *Staphylococcus aureus* by use of the PAAN procedure.

The use of PAAN derivatives for identification and/or quantitation of O-methylated saccharides.

An effective technique for the structural analysis of polysaccharides is the permethylation method, which consists of: (a) isolating the pure polymer, (b) methylating all free hydroxyl groups of the polymer, (c) hydrolyzing the polymer to its component monomeric residues, and (d) identifying and quantitating these residues in terms of type of sugar and position of O-methylation. The PAAN procedure has been extensively used for the final step (step "d") of permethylation analyses. Smiley et al. (29) employed permethylation and the PAAN method to establish the degree of polymerization of specific isomalto-oligosaccharides. Bhatnagar et al. (30) employed the PAAN permethylation approach for the structural determination of a new dextran which arose from a bacterial strain (Leucomostoc mesenteroides). The more challanging analyses of the heterosaccharides which have employed PAAN permethylation techniques include: carrageenan (D-galatose, 3,6-anhydro-D-galatose) by Matulewicz and Cerezo (31), xanthan gum (D-mannose and D-glucuronic acid) by Cadmus et al. (32), and an arabino-galactan from Larix sibirica by Karacsonyi et al. (33).

Recent modifications and observations of the derivatization conditions of the PAAN procedure.

Both the oxime-formation step and (especially) the peracetylation step of the PAAN procedure are catalyzed by pyridine, or pyridine-like compounds -- therefore, the pyridine acts as both solvent and catalyst. Studies by Conners and co-workers (34, 35) demonstrated that both N-methylimidazole (NMIM, also known as 1-methylimidazole) and 4-dimethylaminopyridine (DMAP) could effectively increase the reaction rate (relative to pyridine) of acetylations of hydroxy and poly-hydroxy compounds. McGinnis (36, 37) employed NMIM as both a solvent and catalyst (totally replacing the pyridine) to achieve much shorter total reaction times for the two steps (10 min relative to 40 min for pyridine), but with the potential problem of additional minor GC components (apparently the side products of the saccharides) appearing near the solvent front of the chromatogram. More recently, Guerrant and Moss (38) have employed the solid DMAP as a catalyst in a pyridine/methanol solvent system for the PAAN procedure. In an additional variation on the general PAAN approach, Fanta et al. (39) employed ethyl acetate, rather than chloroform, in the extraction procedure. Furneaux (40) investigated the identity, and quantitative ratio, of side-products from the PAAN

derivatization procedure. By physical isolation, via thin-layer chromatography, Furneaux concluded that a typical hexose (he studied D-glucose and D-galactose) yielded about 70% of the PAAN product and also produced a 30% yield of other products -- essentially the alpha and beta isomers of the pyranoside and/or furanoside products of N-hydroxy-D-glucosamine hexaacetates. The existence (or potential existance) of these side-products have obvious implications for the quantitative aspects of the PAAN procedure.

Recent modifications in chromatographic conditions for the PAAN procedure.

The original GC conditions for PAAN derivatives involved relatively-polar liquid phases (e.g., butaediol succinate) for interaction with the less-polar, partially O-methylated products from permethylation. Later, less-polar liquid phases with higher boiling ranges (7, 8) allowed derivatives of the non-O-methylated sugars and corresponding disaccharides to be chromatographed - OV-17 (a silicone polymer)is a typical example of such a liquid phase. Recently, OV-225 packed columns have been used to separate PAAN derivatives of partially O-methylated galactoses (41) and glucoses (42). Kostenko and Senchenko (43) studied PFMS-6 (apparently a Russian variant of

OV-225), but found it to be inferior to OV-225 -- in this case both liquid phases were employed for the non-O-methylated derivatives. Capillary-column separations by GC have been performed by Freer (44) with deactivated Carbowax 20M, by Guerrant and Moss (38) with OV-1, and by Willis (45) with OV-1 and SP-2100--in each case on coated fused-silica columns.

Analogues and extensions of the PAAN/PAKO derivatizations procedure.

The major features of the PAAN derivatives are (a) the nitrile group which replaces the original aldehyde group of the sugar, and (b) the O-acetyl groups which replace the original hydroxy groups of the sugar. Several variations of this basic derivatization, followed by chromatography of the product, have been reported. Derivatives corresponding to the PAAN compounds, but with the nitrile group replaced by the O-methyloxime group, were employed for GC analysis by Mawhinney et al. (46). Thompson (47) employed analogues with the nitrile groups substituted with the benzyloxime and the acetyl groups replaced benzoyl groups -- with separation by high-pressure liquid chromatography. D'Accoro and Thiel (48) retained the nitrile group, but replaced the acetyl groups with benzoyl groups. Schweer (49) replaced the

nitrile group with an O-butyloxime group, and the acetate groups with trifluoroacetate groups. Shaban et al. (50) replaced the nitrile group with a 1-hydrazinophthalazine group, and retained the acetyl groups. However, (a) these various derivatives have not been employed for free sugar analysis (and rarely for any form of saccharide analysis), and (b) there is no claim, nor any indication in the experimental procedure, that these derivatives are superior for GC and/or GC-MS analysis of saccharides.

Several approaches have been made to incorporate N-acetyl-hexosamines and uronic acids into the PAAN derivatization scheme - compounds which are intrinsically more polar than the neutral saccharides, and which therefore produce derivatives which elute with greater difficulty from the GC columns. Mawhinney et al. (51) used a combination of PAAN derivatives (for neutral sugars) and methyloxime acetates (for the amino sugars) for a coordinated analytical approach. Turner and Cherniak (52) used somewhat more vigorous PAAN conditions to produce both the combined neutral and amino sugars, and chromatographed them on a single column. Lehrfeld (53) employed a modified (pre-reduction) alditol acetate procedure and compared these results to a conventional PAAN analysis -- the difference in yield between the two procedures was the defined at the content of the various

possible uronic acids.

In summary, the above sections indicate a vigorous development and application of both the PAAN procedure, and the investigations of analogous derivatization procedures. Several features are evident in this survey of the literature. Firstly, no procedure has been found which is more effective than the PAAN/PAKO procedure for sugar analysis. Secondly, there are few applications of the PAAN/PAKO procedure for analysis of ketoses and/or disaccharides. Thirdly, the PAAN/PAKO procedure is not employed for the direct analysis of free saccharides from plant tissues. And fourthly, the employment of internal standards and/or electronic integration is rare for any form of quantitation of saccharides *via* chromatographic analysis.

ACCURACY AND PRECISION OF THE QUANTITATIVE METHODOLOGY

The studies within this chapter are an extension of those reported in the Master's Thesis of Stacy Unruh (1), and are also based upon graduate research studies done at SDSU by R. Pietz(64). The major interest in this chapter is the establishment of the level of confidence for the final numerical values reported by the integrator (processing the signal from a GC intrument) in relationship to the actual original wet-weight percentages of free saccharides within a plant tissue under study. The studies reported herein differ from those of Unruh (1) insofar as (a) all studies were done under a more uniform extraction, derivatization, and chromatographic procedure (principly based upon Unruh's final optimized conditions) and (b) the use of sophisticated electronic integration for processing the output of the flame ionization detector (FID) of the GC instrument.

The original quantitation studies of free sugars in plant tissues by Unruh(1) indicated that the PAAN/PAKO procedure was reproducible to within a few percent of the sugar content present. These studies were based both upon repetitive injections of the same derivatization mixture, and also upon injections from parallel derivatizations of the same starting extract sample. Unruh also established

that under the currently employed procedure a region of linearity existed between sample injection size and chromatographic peak-area for samples in the 1.0 ug to 20 ug size. Electronic integration was not available when Unruh's(1) studies started, and the original quantitation studies were made by cutting and weighing of peak-areas from strip-chart recorders. Obviously, such labor intensive operations allowed less extensive studies to be done than are now feasible with electronic integration. Later in Unruh's study an early-model digital integrator (a Varian CDS 111) was used, but extensive quantitation studies were not performed with that instrument. R.Pietz (64) reproduced certain aspects of Unruh's quantitation studies of the PAAN/PAKO procedure using modern recording integrators (a Hewlett-Packard model 3380, a Spectra-Physics model SP4270 and a Varian Vista 401 system). The quantitation aspects of Pietz's (64) research proved frustrating -- although the recording integrators greatly improved the ease of data processing, the reproducibility of repetitive injections of the same sample was bad, with deviations of up to 30% in reported peak-area. Extensive modifications of the default program (for packed column GC analyses) failed to improve the reproducibility of the integration of those chromatographic peaks. The origin of that irreproducibility is not currently understood,

but this study presents a solution which eliminates the problem. The solution simply consisted of changing but a single parameter of the chromatographic program --- increasing the temperature program from 10°C/min to 20°C/min. This temperature-program increase resulted in little loss of peak resolution, shortened the time for each chromatogram by ten minutes, and was responsible for the close agreement of the following data dealing with reproducibility of repetitive injections. The following studies therefore combine: (a) the basic analytical procedure of Unruh, (b) several technical modifications for ease of sample manipulation(described in Appendix A), (c) the faster GC temperature program, and (d) the use of recording integrators for ease of data manipulation and storage.

This study is highly dependent upon the use of internal standards and upon the use of well-defined, reproducible reaction conditions. The internal standard allows convenience of not requiring precision in the chloroform/water extraction steps and/or in the injection procedure, and also provides for automatic compensation should the derivatization steps not be complete. In fact, it is certain that the derivatization steps are not quantitative for the following reasons. Firstly, non-interfering peaks of minor side-products from the

derivatization of aldoses are seen in the chromatograms. Secondly, this lack of total reaction has been observed and discussed in the literature(40). Thirdly, when based upon the original amount of starting material, the chromatographic peaks for derivatives of aldoses and ketoses have FID detector-responses that are distinctly smaller than those for the corresponding peracetylated polyalcohols (e.g., the derivative of D-glucose vs. the derivative of sorbitol -- see Appendix B). It is easiest to rationalize the above low detector-response for the PAAN/PAKO derivatives in terms of the formation of side-products which do not appear under our chromatographic conditions. Also, it is important to recognize that four reactions are involved for the quantitation of any sugar when an internal standard is employed. The first two reactions occur in the calibration solution, these being (A) the derivatization of a known quantity of a reference compound, and (B) the derivatization of a known quantity of the sugar under study. The second two reactions occur in the solution of the unknown, these being (C) the derivatization of another known quantity of the reference compound, and (D) the derivatization of the unknown quantity of the sugar under study. If the conditions (reaction, extraction, and chromatographic) are the same for the two reaction sequences, then the relationship

major potential free-sugar constituents in plant tissues, and also are representatives of the major types of free sugars to be studied, that is: aldoses, polyalcohols, ketoses, and non-reducing dissaccharides. The retention times and detector responses of these and analogous saccharides (when analyzed under conditions described in Appendix A) are listed in Appendix B.

The use of the PAAN derivative of L-rhamnose was continued from Unruh's (1) study. L-rhamnose has the potential problem of producing a minor by-product in the sorbitol derivative chromatographic region. However, L-rhamnose did serve as a useful reference in Unruh's (1) studies, has the advantage of being a true sugar, and therefore, should undergo the same type of reaction (or incomplete reaction) as the unknown sugars. In essence, the existance of this peak in each determination provides positive evidence that both steps of the derivatization procedure proceeded correctly.

The term "percent reproducibility" employed in this study refers to the variation in the area count of a chromatographic peak under consideration (e.g., that peak representing D-glucose) relative to the area count of the peak representing the internal standard (i.e., the peak representing L-rhamnose). The quantitation studies below were achieved by varying the concentration of D-rhamnose

so that a 3.0 uL injection of chloroform extract would correspond to 2.2 ugm of L-rhamnose from the original derivatization, or a 4.4 ugm injection of the PAAN derivative of L-rhamnose (on the assumption that all of the derivatized product produced the GC peak). The actual size of the internal standard peak is not normally reported, and once the linearity of the unknown peak (or peaks) is established, the linearity of the unknown to variance in the reference peak can be assumed to be true. Nevertheless, for the majority of the determinations of unknowns in the following chapters a reference peak was used which was within 10% of the area count (1.9 million for the Varian Vista system) used for the studies within this chapter.

All data presented in this chapter were obtained by employing the standard analytical procedure described in Appendix A, and by performing the analyses with a combined Varian Vista(recording integrator)system coupled to the FID output of a Varian 6000 series gas-liquid chromatograph. The reproducibility of this analytical procedure was evaluated by the following five criteria. Firstly, for the agreement of areas from peaks with a specific retention-time which results from successive injections of the same reference solution. Secondly, for the agreement of peak-areas from parallel derivatizations

of the same mixture of sugars of known concentration. Thirdly, for the agreement of peak areas from parallel derivatizations from the same starting plant extract. Fourthly, for the linearity of detector response for successive injections when the reference peak are was held constant, and the amount of derivatized unknown material was varied. And fifthly, for the linearity of detector resonse from successive injections, when both reference and the sugars to be studied (from a plant tissue extract)were held constant, and additional amounts of the non-reference sugars were added to the extract.

Agreement of repetitive injections of the same solution.

The data in Table 1 are results produced by successive injections of the same chloroform extract into the Varian Vista system. The reaction conditions are those described in Appendix A, but the general procedure differed slightly from the normal approach as follows. A single solution of approximately 40mg each of L-rhamnose, D-glucose, sorbitol, D-fructose with 25 mg of sucrose and 1.40 g hydroxylamine hydrochloride was diluted to 10.0mL with pyridine. Two hundred uL of this stock solution was then derivatized for the analysis. The repetitive injections were made with a 10 uL Hamilton syringe equipped with a Chaney adapter set to deliver 3.0 uL.

Each injection therefore delivered approximately 4.4 ug of L-rhamnose PAAN derivative, which produced a reference peak of about full-scale deflection at 512 attenuation (approximately one-third peak height of the A / D conversion range), and an area count of about 1.8 million arbitrary units of the Vista integrating system. The sorbitol peak-area was not corrected for the contribution of the side-product of L-rhamnose, as this was expected to be a constant contribution. The sugar values in Table 1 are hypothetical in both the sense that no actual wet-weight of a tissue ever existed and due to the fact that both the L-rhamnose and the remaining saccharides were obtained from the same stock solution -- eliminating any source of error arising from separate additions of two solvents. Arbitrary normalization factors were programmed into the computer, which accounts for the unrealistically large "percentages" in the table. Nevertheless, the recording integrator responded as if it were processing real samples, and all computations which are normally performed for a sugar analysis were performed here. The results of each run were computed against a constant calibration chromatogram data set, of roughly equal area count. The resulting data was rounded off as shown in Table 1, and both the mean and the standard deviation for each set of repetitive values were calculated. For these

smaller data sets the actual value of s_{n-1} was employed for the standard deviation. The data in Table 1 agree with several independent similar studies which were made. In general, it has been found that the reproducibility of peaks represented by derivatives of aldoses (D-glucose) and ketoses (D-fructose) is less than that of peaks represented by polyalcohols (sorbitol, and for this purpose sucrose).

Agreement of parallel derivatizations of the same stock solution

The identical stock solution and derivatization method described in the above section were then employed for five derivatizations in parallel. These derivatives are described as R9, R10, R11, R12, and R13 in Table 2. The chloroform extract from each derivatization was injected in triplicate, the data rounded off as shown in Table 2, and ultimately both the mean and the standard deviation (again calculated as s_{n-1}) were established for the values of each sugar. It can be seen that the agreement between each triplicate set of injections is about the same as that in Table 1, but that the overall agreement for the total table is somewhat less. This increase in standard deviation values is attributed to a measure of the error introduced during the derivatization

process which is independent of other errors of measurement.

Agreement of parallel derivatizations of the same extract of a plant tissue and optimum evaporation conditions for that extract.

This step incorporates the findings of Unruh's study which concluded that complete and reproducible extraction of free sugars had occurred under the defined extraction procedure. A series of evaporations were performed in duplicate on identical 0.5 mL samples of the same plant-tissue extract. This extract was obtained by performing the extraction method (described in Appendix A) five times on 1.00 gm samples of the petals from a Hawthorne tree (Crategus sp.), combining these extracts, mixing, and diluting to a total of 50mL. All evaporations took place in teflon-capped 13 x 100 mm culture tubes, which were directly used for the derivatization and chromatographic steps (as defined in Appendix A). The following evaporation procedures were used. Condition A: the extract was directly heated on a white glass surface 4 cm under a 250 watt heating lamp for 20 min. Condition B: the extract was placed in an 85°C heating block for 6 minutes, with an air-flow (and partial vacuum) created by asprator vacuum through a 3mm internal diameter outlet

and a 22 gage 2-inch hypodermic needle as an inlet. Condition C: the same as condition B, except the block temperature was 70°C and 14 minutes were required. Condition D: the same as condition B, except the block temperature was 50°C and 22 minutes were required. In each case the evaporation tube was periodically examined and the evaporative process was stopped a few minutes after no trace of liquid could be observed. Each of the above duplicate determinations was chromatographed in duplicate, and the average of the determinations for each saccharide reported in Table 3. These determinations (as wet-weight percentages of the original plant tissue) were referenced to the area count of the peak of the internal standard, and the average count of each reference peak is also shown in Table 3 (uniform amounts were injected with a Chaney adapter).

Inspection of the data in the first four lines of Table 3 for reaction conditions A through D indicates that (a) evaporation in a heating block under reduced pressure is superior to direct IR heating, (b) only marginal differences in wet-weight percentages of sugars are observed when the temperature of the heating block ranges between 50 to 85°C, and (c) 70°C is a good evaporation temperature (in terms of: time required, total area count for the peak of the internal standard,

and area counts of the unknown peaks relative to the internal standard). The above therefore demonstrates the ability of the derivatization system to reproducibly reflect the amounts of sugars in the extracts of plant tissues. For final confirmation of the above findings, the above extract was rederivatized in four parallel determinations under condition C (see the last four lines of Table 3).

Linearity of the detector response to peaks of sugars relative to a constant reference peak.

Two solutions were derivatized according to the procedure in Appendix A. The first solution (solution A) consisted of L-rhamnose monohydrate (45.5 mg, corresponding to 41.0 mg of L-rhamnose), and hydroxylamine hydrochloride (32.2 mg) in pyridine (1.5 mL). The second solution (solution B) consisted of D-glucose (42.1 mg), sorbitol monohydrate (30.7 mg, corresponding to 27.9 mg of sorbitol), D-fructose (66.3 mg), sucrose (81.4 mg), and hydroxylamine hydrochloride (176 mg) in pyridine (1.5 mL). Chloroform extracts of the derivatives of these solutions were then combined, with Langlevy pipettes, to produce injection solutions which varied by known ratios of the L-rhamnose derivative to the derivatives of the four remaining saccharides. The resulting linearity plot

is only dependent upon (a) the accuracy of the delivery of the two stock solutions and (b) the linearity of the "detector response". In this situation the term "detector response" refers to the sum of a variety of effects, including column performance.

The combination of solutions A and B are as follows: for the first injection solution of a dilution ratio 0.25, 100 uL of solution A, 25 uL of solution B, and 875 uL of chloroform were employed. A similar method was employed for the remaining ten solutions, with all injection solutions containing 100 ul of solution A and a final total volume of 1000 uL. The dilution ratios (defined by the uL of solution B divided by the 100 uL of solution A) of the used injection solutions are listed in Table 4. A Chaney adapter was used to inject 3.0 uL of the injection solution, chromatographed in triplicate, and the recording integrator response for each peak being averaged. These averaged integrator values were then normalized to injected amounts of saccharides -- at a concentration (identified for each saccharide in Table 4) within the linear response region of the chromatogram. The amount of each sugar listed in the "Inj" column of Table 4 is based upon (a) the original amount of sugar to be derivatized, and (b) the dilution ratio of the one mL chloroform extract of the derivatization solution. The

"Res" value in Table 4 is the normalized response of the averaged values of the recording integrator for the corresponding peak. The data in the columns of Table 4 are plotted for each saccharide, and are shown in Figures 1 and 2.

Figure 4 illustrates a typical chromatogram for generating the data presented in this chapter, indicating both the stability of the baseline, and the separation of the peaks. The x-axis represents time elapsed (small numbers above the peaks, in minutes) after the injection. This specific chromatogram is a replot of the data set stored on disk, and therefore indicates the baseline which was post-chromatographically added for computation of the peak areas. For enhanced visual inspection of both peak symmetry and baseline stability, these data were plotted at twice the chart speed (2 cm/min) normally employed for the purpose of monitoring -- obviously, the computations are independent of chart speed. The plot for this chromatogram is attenuated at 512, with a maximal attenuation of 1024 and an A/D over-range at 50% above the maximum 1024 plot range. Therefore, the peak for L-rhamnose is at approximately 25% of the full-scale integrating range. This example is one of the 33 chromatograms used for the data in Table 4 and represents the lowest unknown peak areas to reference peak area of

the series. In the later chromatograms, the peaks of the unknowns rose to over 4 times the height of the reference peak. The chloroform injection peak is not shown, but essential baseline linearity occurred at 2.5 minutes. An analysis (on a sugar-by-sugar basis) of the data in Table 4, and the plots in Figures 1 and 2, is presented below.

The PAAN derivative of D-glucose showed a linear response within the 2 ug to 30 ug injection range -- from 0.4 to 6.0 million area counts (MAC) on the Vista system. These weights, and those below, are based upon the weight of starting material. No deviation in linearity was observed at either end of this range.

The peracetylated derivative of sorbitol showed a linear response in the 1.4 ug to 11 ug injection range -- from 0.4 to 4.0 MAC. In the 11 to 20 ug range, a steady increase in the underevaluation of the expected peak area occurred. This deviation is shown in Figure 1, and a correction plot is presented in Figure 3. No linearity problems were seen at the lower limit of the injection range, but the peak of the L-rhamnose side-product will dominate this chromatographic region when the sorbitol injection level drops below 1 ug (and the integrator may then pick this peak rather the sorbitol peak).

The PAKO derivative of D-fructose displayed a linear response in the 10 to 33 ug region -- from 0.4 to

10 MAC. Under-evaluation of expected peak areas occurred both above and below this injection region, with under-evaluation of about 10% at 45 ug (or about 9.0 MAC). Marked under-evaluation (in terms of percentage of the peak area) occurred under 10ug, with 20% under-evaluation at 7 ug, and 25% at 5 ug (0.8 MAC). The most likely explaination for this under-evaluation is irreversible column adsorption of a certain fixed amount of this derivative upon each injection.

The peracetylated derivative of sucrose displayed a linear response in the 4 to 60 ug injection range (0.8 to 40 MAC) with no indication of deviation of linearity at the lower end of the range, and only slight deviation to 80 ug injection.

The above studies confirm that it is most useful to work in the upper region of weights of a derivative to be injected. The peak height of the internal reference of the L-rhamnose derivative was near the half-height level of maximum A/D conversion of the integration system. If the injection level drops too low (below 0.4 MAC) then under-quantitation of the D-fructose derivative occurs. Interestingly, a part of the working regions of linearity for D-glucose, D-fructose, and sucrose lie above the maximum A/D level of integration -- apparently some clipping of peak tops has little effect on the total

peak area during the first stages of A/D over-range. It is important to recognize that relatively large amounts of material must be injected before a region of linearity for the PAKO derivative of D-fructose is reached. Early evidence indicates that the region of linearity for D-glucose, sorbitol, and sucrose extends to much lower levels; but the derivative of D-fructose (and presumably all other PAKO derivatives) will be greatly underestimated at this level of injection.

It has therefore been demonstrated that a region of linearity encompassing a decade range occurs for the major sugars expected from extracts of plant tissues, and that there is a general overlap of these working ranges. It is important to recognize that the actual percentage of sugars in plant tissues is not important in this study -- the ratio of the amount of internal standard to extracted sugars can easily be varied, and the recording integrator readily programmed to accomodate this change. As typical ranges of sugars in the tissues of plants lie in the 0.5 to 5.0 percent wet-weight range, a single chromatographic determination can quantitate all unknown sugars to within a few percent of their actual values.

Linearity of the FID detector response when both internal reference and the saccharides from a tissue extract are of constant weight, and various amounts of a known sugar are added before dilution.

This study differs from the previous linearity study insofar as: (a) the additional sugars are added before the derivatization procedure, and (b) additional non-sugar components from the plant tissue are presumably present in the ethanol extract. Therefore, this procedure is testing the linearity of the derivatization procedure, with potentially interfering compounds present during the process. The same stock plant-extract used for Part 3 (e.g., petals from a Hawthorne tree) was employed herein. A comparison solution of known sugars in 90% ethanol (100 mL) was produced -- the sugars used were D-glucose (89.7 mg), sorbitol monohydrate (100.1 mg, corresponding to 91.1 mg sorbitol), D-fructose (159.1 mg), and sucrose 22.9 mg). The first test solution was made by combining the Hawthorne extract 500 uL with the above comparison solution (200 uL) -- the actual amounts of free sugar added being: 179 ug D-glucose, 182 ug sorbitol, 318 ug D-fructose, and 46 ug sucrose. Three additional test solutions were made, keeping the amount of Hawthorne petal extract constant (500 uL) but increasing the amount of the comparison solution (400 uL for the second test

solution, 600 uL for the third test solution, and 800 uL for the fourth test solution). Upon combination and evaporation of these solutions, each test solution was derivatized (in duplicate) and analyzed by the procedure in Appendix A. The results of the parallel determinations were averaged, subtracted from the previously established saccharide content in the Hawthorne petals, and compared to the known starting amount of added saccharide from the comparison solution -- a tabulation of these results are in Table 5. The amounts subtracted from the detector response were those determined in Table 3 for Condition C "normalized to the actual amount injected". The actual "amount of injected material" described as "Inj" in Table 5 is based on the starting amount of underivatized sugar, and presumes both total derivatization and extraction into 1.00 mL of chloroform. As an internal standard is employed, neither of the above assumptions is necessary to demonstrate the essential linearity of the amount of extra starting material added to be derivatized to the actual amount present as determined by the recording integrator. In a similar manner, the detector response, designated "Res" in Table 5 depends on the normalization step performed on the data in Table 4, but is independent of the linearity of the relationship under study herein. The tabulated data in Table 5 is plotted in Figures 5 and

6 to indicate the essential relationship of linearity for each saccharide.

In summary, the combined results of the above investigations indicate that the procedures described in Appendix A: (a) represent and optimized approach for the analysis of free sugars in plant tissues, (b) exhibit a linear response within as acceptable percent wet-weight sugar range, and (c) can achieve reproducibility with standard deviations within 0.01 wet-weight percentage. However, the usefulness of this level of analytical predictability is only meaningful within the context of (a) the actual wet-weight percentages of sugars in plant tissues, and (b) the range of such sugar percentages within similar tissues of (seemingly) identical plants -- questions which are addressed in the following chapter.

APPLICATION OF THE GC METHODOLOGY TO PLANT TISSUES

The optimized analytical methodology developed in Chapter 2, and summarized in Appendix A, was employed to determine wet-weight sugar percentages (for simplicity referred to as "sugar-percents")of typical plant tissues. Two fundamental questions were addressed: (a) what sugar percentage range can be anticipated for similar plant tissues, taken at identical times, from plants growing in (apparently) identical conditions, and (b) what are typical ranges of sugar percentages on a day-by-day basis for the above plant tissue samples. The generation of these data are necessary, both to establish a rational sampling technique, and also to establish whether the accuracy of this procedure (estimated at +/- 0.02%) was comparable with the actual wet-weight percentage levels found in typical plant tissue. All plant tissue samples were taken between 1 and 2 pm (Central Daylight-savings Time), and were analyzed as described in Appendix A.

Agreement of wet-weight percentage contents of free sugars of plant tissues from identical sources, but from different individual plants.

This section investigates, on a preliminary basis, the agreement of analyses of the free-sugar content of

extracts from similar tissues (collected under analogous conditions) from different individual plants of the same species. An attempt was made to maximize sample uniformity by (a) using petals from flowers (preliminary data indicated these tissues to be more saccharide-stable than leaves or stems), (b) using a woody plant with a well-defined root system, and (c) using plants growing in uniform soil and lighting conditions. The petals from the wild rose (Rosa arkansana) were chosen, with three individual plants being sampled -- petals from three flowers from each plant were commingled. Three mature plants (each plant with a total biomass of approximately 500 gm) growing at 8 meter intervals and equidistant (one meter) from east-west railway tracks (in Brookings, SD) were chosen, and identified as plants 1, 2, and 3. The plants were sampled in bright sunlight at approximately 18°C, 12 hours after recieving 2 cm of rain, and at approximately mid-term in the flowering season (June 26). Two aliquots (of 500 uL each) were derivatized from each extract, and the chloroform extract from each derivative was chromatographed twice -- these data are summarized in Table 6 and in Figure 7. An examination of the data in Table 6 indicates that the variation of the average of each determination is about the same as the variation of the individual replicate determinations for a given

tissue extract. For example, the average standard deviation for the three sets of values (the underlined values in Table 6) for D-glucose and D-fructose is essentially the same (0.031) as the average standard deviation for the two sets of four determinations each for plant 1 for these two sugars (0.039). The data of Table 6 are plotted in Figure 7, with the vertical bars for each determination representing a +/-0.02% wet-weight deviation (about equal to two standard deviation units). It is therefore concluded that the analytical methodology is at, or near, the optimum reproducibility of sugar wet-weight percentage differences between similar tissues of different individual plants.

Changes in the wet-weight percentage contents of free saccharides of plant tissues from identical sources as a function of time.

As a continuation of the above study, the sugar percentages in the stem of a small vascular plant, the dandelion (Taraxacum officinale) were followed as a function of time. Two areas one meter square (defined as areas 1 and 2), separated by eight meters within a grassy location with full exposure to the sun were chosen. Four samplings occurred over a 5 day interval (starting June 27, 1986) with three individual plants taken from each

area for a single plant extract. The first two days were cool (average daily temperature of about 16°C) and dark with rain. A one day sample lapse occurred, followed by two very sunny warm (about 26°C) days. Two aliquots from each extract were derivatized, and each derivative chromatographed twice (see Table 7 and Figure 8). An example chromatogram for the dandelion stem is shown in Figure 9. Note the peak following D-glucose has a retention time equal to myo-inositol, and this split peak is presumed to represent isomers of that compound. Figure 8 presents the sugar percentage data for areas 1 (dashed line), for area 2(dotted line), and for the average value of areas 1 and 2 (solid line) for each observed sugar. The vertical bars for these data points reflect the magnitude of two standard deviations. An inspection of the data for the sugar percentage for D-glucose indicates (a) differences in values from different areas (even though the tissues from three plants from each area were averaged) are much greater than limitations of the analytical technique, (b) differences of the day-to-day averages from the sugar percentage values (about 40%) can exceed the error of the method (about 0.02%) by more than a magnitude, and (c) the percentages from one area (area 2) consistantly are larger than similar values from the other area. For these data the consistancy of increased

saccharide percentage values for area 2 is maintained for D-glucose and D-fructose (except for a single D-fructose value on day 2) -- apparently reflecting differences in growing conditions between these seemingly identical areas. An inspection of the data in Figure 8 indicates extensive detail (relative to the level of confidence of the determinations) for sugar percentages -- both for comparison of different sugars on the same day, and also for the comparison of such changes on a day-by-day basis.

Therefore, the data in Tables 6 and 7 indicate that the limits of confidence for the sugar wet-weight percentages are below the levels needed for meaningful interpretation of analyses of plant tissues. In addition, the above limited data on replicate determinations from two well-separated growing areas suggest that meaningful general trends for sugar percentages in plant tissues can be established without extensive replicate (for different growing areas) sampling. These observations will be expanded in following chapters with similar data taken from stems of other species of vascular plants on identical days to the above study in Table 7.

COMPARISON OF VARIOUS PAAN/PAKO DERIVATIZATION METHODS.

Several recent articles suggest that the PAAN/PAKO procedure described in Appendix A could: (a) under-quantitate saccharides, or (b) be performed more quickly through use of a suitable catalyst. This chapter investigates these possibilities.

Furneaux's (40) study of cyclic by-products from the PAAN derivatization procedure was an area of concern, not because of the loss of sugars (which is compensated for by the reference solution and internal reference standard correction), but due to the concern regarding conpleteness and reproducibility of the reactions for the various types of compounds under consideration (i.e., aldoses, ketoses, and polyols). For this study it was assumed that inefficiency of the analytical procedure would result in under-quantitation of the reported wet-weight percentages of sugars from plant tissues (or such computations of synthetic solutions representing extracts of these tissues). Therefore, in this chapter an "improved" method is operationally defined as a method which increases the chromatographic peak areas for a given amount of starting saccharide. To avoid confusion with (potential) partial derivatization of the internal standard, all samples were injected with a 10 uL Hamilton

701 syringe equipped with a Chaney adapter and the area counts of the chromatographic peaks were then directly compared. It should be noted that the same amount of starting ethanol-sugar solution was used through-out the experiments in this chapter, and therefore, the absolute values of the data in Tables 8 through 12 can be directly compared. Except where specific modifications are noted, the analytical procedure is that described in Appendix A. Two stock solutions were employed for the first step of all studies in this chapter: the first (an ethanol-sugar solution) was evaporated to dryness at 70°C under reduced pressure and the second (a pyridine solution) was added to start the reaction. Solution A (500 uL used) consisted of L-rhamnose monohydrate (130 mg), D-glucose (126 mg), sorbitol monohydrate (79.7 mg), D-fructose (155 mg), and sucrose (231 mg) diluted to 100 mL in a volumetric flask with 90% ethanol. Solution B (200 uL used) consisted of hydroxylamine hydrochloride (1.49 gm) diluted to 10 mL in a volumetric flask with pyridine.

Comparison of GC peak areas to changes in the reaction time for the first (oxime formation) reaction step.

Stock solutions A (500 uL) and B (200 uL) were used according to the procedure in Appendix A -- starting with the extract evaporation step. Four reaction

conditions were employed (with duplicate determinations) with changes in reaction time for the first step of 10, 15, 20, and 25 minutes. The normal reaction time in Appendix A is 20 minutes for this step. In all cases the reaction time for the second step was 30 minutes. These data are presented in Table 8, and an inspection of the data indicates that equilbrium for the oxime-formation step has essentially been reached after ten minutes. It could be noted that the data in Table 8 (in terms of peak areas for repetitive injections) are quite uniform -- indicating that reasonably quantitative data can be obtained without the use of an internal standard if a Chaney adapter is employed.

Comparison of GC peak areas to changes in the reaction time for the second (acetylation) reaction step.

The same stock solutions (A and B) and analytical conditions (from Appendix A) used in the previous section were employed in this section -- the sole changes herein are in the reaction time of the second (acetylation) reaction step, with times of 20, 25, 30, and 35 minutes. The normal reaction time in Appendix A is 30 minutes for this second step. In all cases the reaction time for the first (oxime-formation) step was 20 minutes. These data are presented in Table 9, which indicate that the second

step reaction is nearly complete at 20 minutes, and is essentially complete at 30 minutes. Importantly, the area count of the peak representing L-rhamnose is increasing at about the same rate as the area counts of the peaks representing the other sugars. Therefore, the percent wet-weight, related to the L-rhamnose internal standard, is essentially constant for the four remaining sugars under all reaction conditions tabulated in Table 9. It should be recognized, by comparison to the level of replication established in Table 2 and by the fact that these data were obtained with a Chaney adapter, that differences in the last two lines in Table 9 are essentially statistically insignificant.

Comparison of three PAAN/PAKO derivatization procedures.

The literature survey of Chapter 1 has identified two recently used procedures for PAAN/PAKO derivatization which employ catalysts. The first alternative method is that of Chen and McGinnis(36)which uses N-methylimidazole (NMIM) as both the solvent and catalyst, with both of the reaction steps requiring a total of 10 minutes. The second alternative method is that of Guerrant and Moss (38) which uses the solid dimethylamine pyridine (DMAP) as a catalyst dissolved in a pyridine/methanol solvent. Chen and McGinnis only evaluated their method (referred

herein as the NMIM method) for the derivatization of aldoses, whereas Guerrant and Moss studied the products of both aldoses and ketoses with the DMAP method.

To evaluate the above three procedures the same stock Solution A as described in the preceding section was evaporated (500 uL). The NMIM and DMAP methods were used as described in the original literature (36, 38), and the pyridine method as described in Appendix A. For uniformity, the final reaction mixtures for all 3 methods were extracted with chloroform, and chromatographed as described in Appendix A. These data are summarized in Table 10. A comparison of the data in Table 10 indicate that the results of the pyridine procedure and the DMAP method are essentially identical. The DMAP method has the nominal advantage of a shorter reaction time, but when compared to the results for shorter reaction times for the pyridine procedure (see Tables 8 and 9), the actual catalytic effect of the DMAP appears to be marginal. It should be noted that the solvent for the DMAP approach is pyridine, and the reaction temperatures are essentially the same as for the pyridine method.

A comparison between the pyridine method and the NMIM method suggests that the latter approach has serious defects. Firstly, the reaction conditions apparently do not allow reaction completion, based on the reduced area

counts for all peaks in the chromatograms from the NMIM method. This incompleteness of reaction is only a minor problem, insofar as an internal standard can be employed. A more serious problem is the severe under estimation of D-fructose, relative to the aldose derivatives -- though again an internal standard, coupled with an appropriate detector response factor , could reduce this problem. We confirm what Chen and McGinnis (36) noted -- that extra prominent GC peaks (of unknown origin) occur in these chromatograms. The extra peaks occur between the solvent front and the region for the pentoses, giving rise to confusion with regard to the presence of such starting sugars in the original derivatization mixture. Finally, it should be noted that the ratio of side-product to major peaks for aldoses (as observed from the area counts of the peaks from D-glucose) is essentially the same for all three methods.

Modification of the acetylation step of the NMIM method by increasing the temperature and/or the reaction time while moderating with a pyridine solvent.

In contrast to the DMAP method, which is actually very similar to the pyridine procedure, the NMIM method (which occurred at ambient temperature) held promise for modification. The first reaction modification consisted

of raising the temperature of the NMIM procedure, which resulted in a black reaction mixture yielding a complex chromatogram. The second reaction modification consisted of using reaction conditions for the first step identical to those of the pyridine procedure (in Appendix A). The second step was also the same as in Appendix A, except that (a) 20 uL of N-methyl-imidazole was added with the acetic anhydride (creating a 90% pyridine / 10% N-methyl-imidazole catalytic ratio), (b) ambient conditions were used in the first trial, and (c) varying reaction times at 70°C were employed. Each reaction condition was performed in duplicate, with the results being summarized in Table 11. The detector responses for each saccharide in line 3 of Table 11 (representing conditions of a 20 minute reaction time at 70°C) are essentially the same as those of our pyridine method of Appendix A (compare to line 3 of Table 9). The data in line 2 of Table 11 (representing similar reaction conditions with a 10 min. reaction time) indicate incomplete peracetylations. In the chromatograms from all reaction mixtures which used NMIM as a catalyst the undesired extra chromatographic peaks occurred near (within 4 minutes) of the solvent front. It is therefore concluded that these conditions hold little, if any, advantage over the procedure in Appendix A.

Modification of the acetylation step of the NMIM method by increasing the percentage of N-methyl-imidazole in the solvent.

A logical alternative to the modification of the NMIM procedure described above consists of changing the NMIM / pyridine catalytic ratio. The only modification from the above procedure, with stock Solutions A and B and Appendix A procedure, was the addition of measured amounts of N-methylimidazole (from 25 uL to 200 uL) with the acetic anhydride at the start of the second reaction step. The addition of 200uL of NMIM, to give the highest NMIM / pyridine ratio (1:1), produced a black reaction mixture which yielded a chromatogram with complex, multiple peaks. The results of the chromatograms from the remaining reaction modification conditions are shown in Table 12. For this section the reaction temperatures for both steps in all conditions were 70°C. In essence, the data in Table 12 indicate that at 70°C increasing percents of NMIM in the solution of the second reaction step have little effect on the rate of peracetylation, and percentages near or above 50% (NMIM to pyridine) are detrimental to saccharide quantitation.

In summary, the conditions described in Appendix A represent the optimum conditions that have been found for accurate quantitation of saccharides by the PAAN/PAKO

procedure. No modifications of this PAAN/PAKO procedure (either as described in the literature, or a logical variations of these procedures) are known to yield equivalent results.

THE FREE SUGAR CONTENT OF THE TISSUE OF CANADA THISTLE

Chapter 3 concluded with the analysis of the free sugars in the stem tissue of the dandelion. This chapter expands such analyses to the stem tissue of the Canada thistle (Cirsium arvense). The data within this chapter can directly be compared to the previous dandelion (Taraxacum officinale), and also to the data in the following chapter for field bindweed, in the following senses: (a) the same analytical method (Appendix A) was used, (b) the plants were sampled at the same time on the same day (day 1 corresponds to June 27,1986 in all cases) in the same general vicinity, (c)the stem tissue was used and (d) the sampling method (two defined areas 8 m apart with three plants from each area for each extract) were the same. The transverse study(in terms of time) occurred on 4 days (2 dark and 2 bright) over a 5 day interval -- see Table 13 and Figure 11. A typical chromatogram, upon which the data in this table and figure are based, is shown in Figure 10. A comparison of the data in Figure 8 (for dandelion) and in Figure 11 (for Canada thistle) reveals that D-glucose is the dominant free sugar for dandelion stem tissue, whereas sucrose is the dominant free sugar in Canada thistle stem tissue.

In the course of the above studies on vascular plant tissues, an undergraduate research project was done by Nathan A. Anderson (4) which applied the general method of Appendix A to the leaf tissue from nine species of trees, each species representing a major family of decidious tree. Evidence from both this study, and from that of Unruh (1) also indicated that all non-petal plant tissues of wood trees, including the stems, have about the same saccharide content. Anderson's (4) data on the wet-weight saccharide percentages of leaves of trees can be summarized as follows: (a) sucrose is always the dominant free sugar, with typical values in the 1.5% to 4.0% range, (b) D-glucose rarely occurs (in 3 of the 9 studied species) and a typical range is 0.3% to 1.0%, (c) free D-fructose did not occur, and (d) all identifiable sugars have elevated sugar percentages on bright days.

Not only are the data from the stem tissues of the Canada thistle and the dandelion different from each other, these data also differ form the tree leaf data (and presumably the analagous stem content of these species). These vascular plant examples are typical of a preliminary survey of the saccharide content of a dozen different vascular species, data which indicate that typical wet-weight saccharide percentages (for any given saccharide) are typically under 1.0% and are often under

0.4%. Therefore, the trends of saccharide content for any given day are quite different for vascular plant-tissue when compared to woody-plant tissue. Although depressed (by about a factor of 5 compared to woody-plant tissue) the sucrose content of vascular-plant tissue does on a day-by-day basis, parallel the pattern favored by the leaves of trees -- that is, the sugar percents increase on bright days. The inositol content of the Canada thistle was also followed, although not plotted -- in general, this content remained constant at about 0.13 wet weight percent (a value which approximates the inositol content of the dandelion).

A total survey of the literature relating to Canada thistle (Cirsium arvense) was performed for the past twenty years (1965-1986; principly based upon the Science Citation Index). All identified articles were obtained and read, and all references in those articles which referred to carbohydrates in any context were obtained and read. A total of 58 papers were examined, and it was established that the great majority of the articles dealt with: growth, distribution, reaction to insects, and/or reaction to herbicides. No references to inositol in any plant tissue of Canada thistle was found, and only three references to "carbohydrates" in general were located. Two references referred to seasonal

fluctuations of "water-soluble carbohydrate" in the root system of Canada thistle - that is, non-structural, water soluble saccharides which act as energy reserves. The earliest paper by Otzen and Koridon (54) concluded that the "water-soluble carbohydrate" content, on a wet-weight percentage basis, could vary from a low of 4% (for April in the Netherlands to a high of 24% (in November) -- in all cases the analyses were performed after extensive hydrolysis of the extract. Otzen and Koridon surveyed for free "reducing" sugars in the root tissue, by use of a traditional titration method using copper reduction, and concluded that the total reducing sugar content of the roots was less than 0.5 percent and relatively stable on a yearly basis -- data in accord with the findings of this thesis. Later McAllister and Haderlie (55) did similar studies on root buds of C. thistle in Nebraska, but did not do any parallel studies on reducing sugar content. The single remaining reference to C. thistle and carbohydrates was by Wallace (56), who identified rhamnose and glucose as hydrolysis products (by paper chromatography) liberated from flavonoids which had been extracted from the thistle.

The above specific example for references to free sugars from tissues of C. thistle is typical, in terms of paucity of material, for common vascular plants. It could

be noted that Cirsium arvense, the so-called "Canada thistle", is not native to North America, being a relatively recent import from Western Europe, and reaching the Northern American plains in the late 19th century. Therefore, as a prominent noxious weed, Canada thistle has been extensively studied in the United States Canada, and Europe -- it is not an obscure species.

THE FREE SACCHARIDE CONTENT OF FIELD BINDWEED TISSUE

As a compliment to the study of the stem tissue of the dandelion and the Canada thistle, the saccharide content of the stem tissue of field bindweed (Convolvulus arvensis) was examined. The days involved in sampling, the methodology, and the analytical method were identical to the previously discussed data from dandelion and Canada thistle. In fact, the defined areas 1 and 2 for the field bindweed and the dandelion are identical -- the Sexauer sports-field of SDSU. A typical chromatogram for derivatives derived from the stem of field bindweed is shown in Figure 12, and the resulting transverse data are shown in Table 14 and Figure 13.

The sucrose content of the stem-tissue of the field bindweed is quite similar to the saccharide content of corresponding tissue from dandelion and Canada thistle -- both in percentages on any given day, and also in the "profile" of the transverse day-to-day sugar percentage studies. However, both the D-glucose and the D-fructose data for stem-tissue from field bindweed differ radically from the corresponding data for dandelion and Canada thistle -- with D-fructose being essentially absent from the field bindweed tissue. Upon exposure to bright sunlight, there was a sharp increase in the wet-weight

percentage for D-glucose in field bindweed tissue. In general, these data from field bindweed much more closely parallel the data obtained from the leaves of trees (4) than they relate to data from other plants presented in this thesis. In addition, less "coherence" in the high and low (relative to the average) saccharide values for the corresponding saccharides of the two sampling areas are observed for the field bindweed (e.g., for the dandelion data, essentially all saccharide values for a given day from Area 2 are greater than those values for the corresponding Area 1). Possibly this lack of wet-weight percentage coherence between the two areas results from only an eight-meter separation of the two sampling areas and from the more-extensive root system of the field bindweed.

In a manner analogous to the Canada thistle, the literature for the field bindweed, an allied species, was sought through the Science Citation Index. A generalized search for Convolvulaceae (which includes field bindweed, hedge bindweed, etc.) produced a total of 176 articles -- 35 articles specifically dealing with field bindweed (Convolvulus arvensis). A total of seven of these articles referred to "carbohydrates" in any form, and only 3 of these articles related to Convolvulus arvensis. In 1940, Barr (57) examined both the "water-

soluble carbohydrate" and the "reducing sugar" content of the roots of Convolvulus arvensis in Colorado on a yearly basis. Both the methodology and the general results (in terms of wet-weight percentages)were similar to the above cited (54) study of root tissue from Canada thistle. However, the data of Barr for reducing sugar wet-weight percents of field bindweed values in the summer (ranging about 1.3 percent) are considerably higher than the corresponding amount this study finds (about 0.3% as D-glucose) in the stem tissue. A second study, in 1970 on the effect of herbicides on Convolvulus arvensis by Chodova and Zemanek (58) in Czechoslovakia found similar amounts of "water-soluble carbohydrate", but did not examine the free saccharide content. A third discussion of "carbohydrates" in Convolvulus arvensis can be found in the review article by Weaver and Riley (59), which does not expand upon the above cited literature. The remaining four articles for Convolvulaceae in general are even less germaine to this thesis. Two articles on extra-floral nectaries (nectar secreating glands) in species of Ipomoea (60, 61) indicate high free-saccharide wet-weight percentages in the floral nectar. Two other articles indicate D-glucose and D-rhamnose as the hydrolysis products from resins obtained from Ipomoea operculata (62) and from Convolvulus microphyllus (63).

As a species, the field bindweed is similar to the Canada thistle insofar as it is both a recent introduction into North America from Europe, and that it is a noted noxious weed -- again, an example of a well studied plant for which little free sugar data exists.

It can be noted in the chromatogram of Figure 12 that no inositol peak occurs (as found in dandelion and Canada thistle), but that there are two additional prominent peaks (one before, and one after the L-rhamnose peak). In addition, there is a small peak at an retention time (RT) of 9 min, before the sucrose peak. This latter, minor peak is of interest, for in the corresponding chromatogram of the tissue from the flower of bindweed (chromatogram not shown) this peak at 9 minutes becomes a dominant peak. All 3 of these GC peaks are highly unusual for chromatograms from plant tissue, and all 3 components are found (to a greater or lesser extent) in tissues of the flower and leaf of field bindweed. Mass spectrometry (MS) has been employed to examine all peaks in both the chromatograms of the Canada thistle and the bindweed, and on the basis of these studies, the following structures are proposed for these GC peaks.

The first unknown GC peak(RT=2.97 min) represents peracetylated 1-O-methyl erythritol (or threitol) -- see Figure 14a. The mass spectral parameters of both the

N-15 and the deutero-acetylated isotopic substitution analoques of the derivative (for both e.i. and c.i. mass spectrometry) are in accord with the proposed compound -- see the following chapter for a summary of the MS approach that was used. As isotopic substitution confirms that the O-acetyl groups were added during the process of derivatization, it is concluded that the original free saccharide in this tissue was O-methyl erythritol (or the corresponding threitol product -- mass spectrometry does not indicate the stereochemistry of the molecule).

The second unknown GC peak (RT = 4.61 minutes) exhibits the mass spectral parameters associated with a peracetylated pentitol. Of the possible stereoisomers of the structure, only the derivative of D-ribitol has the same retention time. Therefore, it is concluded that the original free component in field bindweed tissue is ribitol, though the MS data does not indicate whether the D or L isomer is involved.

The third unknown peak, with only a trace amount present in the stem-tissue, (RT = 9.43 minutes) has mass spectral properties which indicate a complex glycoside. The MS component of the glycon is identical to that from complex glycosides containing one glucopyranosyl residue which is attached through the reducing group. The MS component of the aglycon group is essentially identical

to that of the MS contribution for the first unknown peak in the chromatogram. Therefore, it is proposed that the parent compound for this per-O-acetylated product is a 1-O-methyl erythritol glucopyranoside (see Figure 14b), or a stereoisomeric analogue.

Ultimately, it should be noted that free ribitol is rarely, if ever, found in plant tissues. In addition, a search through Chemical Abstracts formula index shows that no O-methyl erythritol (or isomeric analogue) nor corresponding complex glycoside has ever been reported in nature. Though the transverse data for these components has not been plotted, the actual wet-weight percentage of these compounds (using detector response factors from derivatives of similar compounds) remains relatively constant, with the 1-O-methyl erythritol and also the ribitol content approximating 0.04 percent.

STRUCTURAL ANALYSIS BY MASS SPECTROMETRY

A fundamental rationale for the development of the quantitation approach described in Chapter 3 was to provide a complimentary analytical methodology for the sophisticated GC-MS approach developed by Unruh (1) for the rapid identification of new and/or unusual saccharide components in plant tissues. This methodology of Unruh was applied to the derivatives from both Canada thistle and field bindweed which are described in the 2 preceding chapters. All chromatographic components described in those chapters were completely structurally identified by the methodology described by both Seymour (7, 8) and by Unruh (1).

The general strategy developed for the above GC-MS structural determination method consists of first performing ammonia-chemical-ionization/mass-spectrometry (ACI-MS) on the chromatographic run with a non-isotopically substituted derivatization mixture from a given plant tissue. Simple spectra result from each chromatographic peak when ACI-MS is used -- often but a single mass peak of M + 18 (where M is the molecular weight of the derivative of the saccharide and 18 represents the molecular weight of the ammonium ion adduct). Therefore, the molecular weight of each derivative is quickly found.

Two parallel derivatizations are also done on aliquots of the plant tissue extract, one derivatization which uses hydroxylamine (N-15) and another which employs deuterated acetic anhydride -- both additional derivatives are subjected to GC and monitored by ACI-MS. The mass spectra corresponding to each peak in the chromatogram are then cross-indexed and referenced to the m/e of the non-isotopically substituted derivative. If the N-15 m/e increases for a given GC peak, then the compound associated with that peak contains an aldo or keto group, and is probably a reducing saccharide. If for a given GC peak, the increase in m/e upon deutero-acetylation is divided by three (the number of deuterium in each acetyl group) then the number of original hydroxyl groups in the starting saccharide is identified. The above data can provide extensive information upon which to base further structural inferences from the electron impact (EI) mass spectra which is then taken for these derivatives.

The above cited MS studies dealt primarily in terms of monomeric aldoses and ketoses -- mass spectrometers capable of dealing with the high GC temperatures involved in the separation of disaccharides were not then available. However, for complete MS analysis of the compounds involved in the chromatographic studies described in Appendix A, additional MS data is needed on

these larger saccharides -- both for disaccharides and for complex glycosides (a covalently bonded saccharide and a non-saccharide moiety) which could elute in this chromatographic region. Thus, representative examples of these two classes of saccharides were studied by GC-MS; the chromatographic and ACI-MS data being summarized in Appendix B. Due to the relative simplicity of the ACI-MS spectra (principly composed of the m/e = M + 18 peaks) this data can be easily summarized. Note, the dashes in Appendix B refer to NO data being currently available for this compound -- in all studied cases the M + 18 peak was present. The data essentially confirm the basic validity of the use of the M + 18 peak approach for the analysis of data from disaccharides and complex glycosides.

The EI-MS data resulting from these studies are more complex and are divided into two sets. The first data set, dealing with EI-MS from disaccharides, is summarized in Table 15. Although there are a number of interesting relationship between these data, several are prominent. In the first data set, the EI-MS of trehalose (a disaccharide composed of two anomerically linked D-glucopyranoside groups) indicates that an intense m/e at 331 is diagnostic of the D-glucopyranosyl residue. An additional relationship a similarly intense peak at 211 for the anomerically linked D-fructofuranoside residue of

sucrose -- though this is not as diagnostic, due to a weak m/e 211 contribution from the glucopyranoside ring (compare the EI-MS of the trehalose derivative to that of the sucrose derivative). Interestingly, under EI-MS conditions the m/e pattern of the derivatives of the reducing disaccharides, which do have the nitrile group incorporated into the peracetylated structure (see the last three columns in Table 15) are (a) quite similar, despite different linkage-type positions, and (b) have most of their m/e contribution from the fragmentations of the ring rather than the (now) linear reducing saccharide moiety. Therefore, EI-MS clearly distinguishes between sucrose and the reducing disaccharides; but is not a good method to differentiate the reducing disaccharides one-from-another (although of course these derivatives can have different GC retention times.

The EI-MS data from a select set of derivatives of complex glycosides is presented in Table 16. These data indicate that the glycoside moiety of the complex glycoside makes a uniform and predictable contribution to the mass spectra of these compounds, and that this contribution is similar to the m/e fragmentation pattern established for these glycoside units from the above disaccharide studies -- note the 331 contribution of the D-glucopyranoside ring in the last three columns of Table

16. To test this approach to mass spectral analysis, the EI-MS of 2 peracetylated derivatives, methyl-beta-D-xylopyranoside and methyl-beta-D-glucopyranoside, were compared (see the first two columns of Table 16). The parallel between the mass-loss of the D-xylopyranoside unit compared to the m/e values of the D-glucopyranoside unit, clearly parallel the difference in mass between these 2 sugars. It is presumed that the molecular weight of other glycon residues can be determined, based on mass differences from the m/e 331 (and allied m/e) of the D-glucopyranoside residue. Once the appropriate contribution of the glycon group is factored from the EI-MS of a complex glycoside, the m/e contribution of the aglycon group can be identified and analyzed. This approach for analysis was taken with the EI-MS of the complex glycoside from field bindweed, and once the familiar pattern of the D-glucopyranoside peaks were removed the resulting spectrum was nearly the same as the mass spectrum produced by the first unknown peak -- which had been identified as 1-O-methyl erythritol.

The mass spectral conditions employed in this study were essentially those reported by Unruh (1). However, in this case all studies were done with a packed GC column under the same GC conditions as described in Appendix A.

CONCLUSIONS

It has been demonstrated that the PAAN/PAKO procedure can be combined with an internal standard and with digital integration to achieve a precise method for quantitation of free saccharides in plant tissues -- conditions which are described in Appendix A. A survey of conditions alternative to the above procedure, either published or logical extensions of those modifications, has failed to find a better approach -- either in ease of application, or in improvement of quantitation.

Importantly, the methodology of Appendix A can be employed for free sugar surveys in plant-tissues from vascular plants. This improved methodology includes appropriate ratios of reagents, control over reaction conditions, and a judicious injection size range for chromatography. Such saccharide surveys of tissues from vascular plants had proviously proven difficult, or impossible, apparently due to (a) a tendency for large percentages of chlorophyll or compounds associated with chlorophyll to inhibit the oxime formation step, and (b) by the low wet-weight percentages of saccharides which are present in these tissues (values of 10 percent of those often found in the leaves of woody plants).

Typical chromatograms which result from the

application of the methodology of Appendix A to tissues of vascular plants are relatively simple -- in terms of the number of peaks present, and the resolution of the GC column. However, these chromatographic peak areas (and on occasion the absence of such peaks) demonstrate: (a) the identity and concentration of the major free sugars (D-glucose, sorbitol, D-fructose, and sucrose) can vary dramatically from species to species, and (b) the large changes in concentrations of these sugars (in a given species) can change extensively on a day-to-day basis -- apparently as a function of growing conditions. As analytical chemistry is the basis of this thesis, no attempt has been made to assign a physiological or a biochemical significance to these findings. Nevertheless, the literature has been searched (for vascular plants in general, and for the dandelion, Canada thistle, and field bindweed in specific) and no comparable data for surveys of wet-weight percents in such tissues have been found.

In addition to surveying for the above listed four major free saccharides, the methodology allows a simultaneous survey for other saccharides which are not extremely polar (e.g., not containing carboxylic acid or phosphate groups), and which have molecular weights less than a trisaccharide (about 400 daltons). No analogs of the major free sugars (e.g., D-mannose with, or in place

of D-glucose), have been observed in the (currently) limited number of studied species, but it is common to find other free sugars. The identity and concentration of these "extra" free sugar components varies widely from species to species. For example, both the dandelion and the Canada thistle stem-tissues contain large amounts of inositols, whereas the stem-tissues of the field bindweed contain appreciable amounts of ribitol, an O-methyl-erythritol, and a complex glycoside incorporating this latter saccharide. The identity of these additional saccharides has been established by GC-MS.

In addition to the above quantitative studies, mass spectral studies (both electron impact and chemical ionization) have been performed on products arising from the PAAN/PAKO methods. For comparison purposes, the MS fragmentation patterns of the PAAN/PAKO(or peracetylated) derivatives of model compounds were studied for complex glycosides and disaccharides. These data were then used for structural confirmation and analysis of the compounds producing the "extra" peaks in the chromatograms of PAAN PAKO derivatizations of plant tissues.

In summary, this thesis defines a precise method for the identification and quantitation (in terms of wet-weight percentages) of free saccharides in the tissues of vascular plants.

APPENDIX A

This section describes and defines the operational steps used for the Master of Science research described in this thesis. Important operations were: (a) tissue extraction, (b) derivatizations of free saccharides, (c) chromatography of the sugar derivatives, and (d) computer instrument control and data processing of the flame ionization detector (FID) chromatographic process. Also included is a description of supporting operations, such as, preparation of internal-reference solutions, and preparation of calibration-reference solutions.

Plant tissue collection and extraction.

The tissue samples employed in this study were collected from their natural environment in the area of Brookings, South Dakota . Upon collection and return to laboratory, the samples were immediately extracted with 90% ethanol. A sample of plant tissue (1.00 gm +/- 0.01 gm) was weighed with a top loader balance in a tared 25 x 150 mm test tube. Ethanol (6 mL of 90%) was added to the test tube, which was then kept in an ice-water bath. The tissue sample-ethanol mixture was subjected to 1) ultra shear disruption (90 seconds at 90% maximum speed) with a Tekmar Tissuemizer, the tissuemizer probe was washed with

ethanol (about 2 mL) which was transferred to the total solution, eliminating a possible source of material loss, and 2) ultrasonic disruption (30 seconds at 35% maximum power) with a Fischer Sonic Dismembrator (model 300). The resulting suspension was centrifuged with a clinical centrifuge (2 minutes at high speed) and the supernatant transferred to a 10 ml volumetric flask. About 2 mL more of 90% ethanol was added to the residue, the mixture was vortexed mixed, centrifuged and the supernatant combined with the previous supernatant in the volumetric flask. The ethanol extract was then diluted to the mark for a total volume of 10 mL and transferred to a teflon capped 16 x 125 mm culture tube and stored at -20°C. About one hour was needed to process a set of six samples of plant tissue. This procedure replaces the more conventional lengthy extraction of saccharides with hot solvents and has the advantages of shear and ultra-sound disruption. Also there is less chance that the extract will degrade. Although this extraction procedure seems very reasonable, a similar approach has not been found in the literature.

Extract preparation.

In order to prepare a PAAN/PAKO derivative from a plant sample, an aliquot of the ethanol plant extract is taken. The preferred aliquot size may vary (between 0.50

mL and 2.00 mL), depending upon the free sugar content of the extract. It has been found that the chromatographic area count of the sugar component is best quantitated when the component's area count was about the area count for the internal standard, L-rhamnose (6-deoxy-L-mannose) PAAN derivative. Due to the higher sugar content of the petals it was usually necessary to take a smaller aliquot (about 0.5 mL) for their extracts than for the extracts of the corresponding leaves and stems. An aliquot of the ethanol extract was taken, using a disposable pipette (1 or 2 mL graduated) to avoid cross-contamination and delivered to a teflon capped 13 x 100 mm culture tube. The extract was placed in a 70°C heating block for about 8 minutes (evaporation was stopped about 2 minutes after apparent dryness was reached) with an air flow (and partial vacuum) created by asperator vacuum through a 3mm outlet and a 22 gage 2-inch hypodermic needle as an inlet.

Derivatization procedure.

After the ethanol extract was dried, a solution (200 uL) of an internal reference (L-rhamnose) was added with a Langlevy pipette. This internal reference solution consists of L-rhamnose monohydrate (e.g., 166 mg corresponding to 150 mg of L-rhamnose) and hydroxylamine

hydrochloride (e.g., 7.54 gm) diluted with pyridine to 50 mL in a volumetric flask. Therefore, 664 ug of L-rhamnose and 30.2 mg of hydroxylamine hydrochloride were added to the reaction mixture. The derivatization proceeded with the addition of a small teflon-coated stirring-bar to the reaction vial, which was sealed with a teflon-lined screw-cap, and placed in a heating block (Pierce Reati-therm heating-stirring module) for 20 minutes at 68°C. After about two minutes of heating the reaction solution was gently swirled over the upper glass surface of the vial to insure that all the evaporated material will be dissolved. Following the first heating step, acetic anhydride (0.300 mL, a volume in excess of the pyridine) was added to the reaction mixture, and the sealed tube again heated for 30 minutes at 68°C. Pyridine (0.100 mL) was then added to readjust the volume to an excess of pyridine.

Extraction of the reaction mixture.

The PAAN/PAKO derivatives produced in the above reaction steps were separated from the reaction reagents as follows. Three test tubes (16 x 122 mm) were arranged in a test tube rack for each extraction. The first tube contained chloroform (1.00 mL) and water (2.00 mL). The second tube contained water (2.00 mL) and pyridine (50

uL). The third test tube contained only water (2.00 mL). The reaction mixture was transferred to the first tube (containing the chloroform and water) by means of a Pasteur pipette, vortex stirred for 30 seconds, and after phase separation the chloroform layer was transferred to the second test tube. The vortex-stirring and chloroform-transferring were then repeated for the remaining wash solutions. If an emulsion formed, phase separation was accomplished by centrifugation. The final chloroform extract was transferred to a clean, dry 13 x 100 mm screw cap culture tube and dried by 30 minute contact with half-dozen pellets of activated (350°C for 3 hours) molecular sieve ("Linde" type 3A, 1/16 inch). To reduce chromatographic solvent front effects the chloroform extract was often concentrated by methods analogous to the ethanol evaporation procedure. If the chloroform extract inadvertantly went dry, it could be redissolved in chloroform (75 uL) without affecting quantitative results. The chloroform extract containing the PAAN/PAKO derivatives was now ready for GLC injection and was usually stable for several months.

Preparation and employment of the calibration reference solution.

Two reference solutions are required for the quantitation of an unknown saccharide(s) in extracts from plant tissue by use of a gas-liquid chromatograph. The first solution, containing the internal reference (L-rhamnose) has previously been described. The second solution, the calibration standard, contains derivatives of known amounts of both the internal standard and also of all saccharides which will be quantitated. A typical calibration reference standard was made by use of a teflon-capped 13 x 100 mm culture tube equipped with a small stirring bar. The derivatization of this standard occurred in the same manner as described above, with the following changes in the amounts of reagents: L-rhamnose monohydrate (45.9mg, corresponding to 41.4mg L-rhamnose), D-glucose (43.2 mg), sorbitol monohydrate (28.1 mg, corresponding to 25.6 mg of sorbitol), D-fructose (61.9 mg), sucrose (79.3 mg), and hydroxylamine hyrdrochloride (189mg) were reacted in pyridine (1.5 mL) and then acetic anhydride (1.7 mL). After the second reaction step, pyridine (0.2 mL) was added, and the typical extraction sequence was employed using chloroform (5 mL) and water (3 x 10 mL). To the second wash solution was added pyridine (0.2 mL). Solutions such as this are stable (in

terms of the resulting integrator count) for more than a year. A typical use of this reference solution is to set the Varian Vista system at an attenuation of 512, and to inject 1 uL of calibration solution yielding essentially full scale peak deflections and area counts in the range of one million.

The above calibration solution is first used on an initial chromatographic run to establish the retention time for each peak corresponding to a given derivative of a sugar to be analyzed and for the retention time of the internal standard -- the fundamental parameters for computations of future unknown chromatographic runs. Then a second chromatographic run is made with the reference solution, and the recording integrator programmed to respond to each specific sugar derivative, as identified by the previously established retention times -- to establish relative response factors (RRF) for each peak.

All recording integrators require specific values for (a) relating specific operational details such as original sample size, and the amount of internal standard and (b)the optimization of the calculation with decisions for peak choice, base-line establishment, etc. The following list identifies the parameters employed for the wet-weight percentage calculations by the Varian Vista system, and also the parameters used for the automatic

```
SINGLE CHANNEL METHOD: CARB 20


SECTION 1: BASIC
PAGE 1
  ANALYSIS PARAMETERS
  CHANNEL: 1
  CALCULATION: IS
  AREA/HT: A
  STOP TIME:  12.00
  NUMB EXPECTED PKS:   40
  EQUILIBRATION TIME:  0
  UNRETAINED PK TIME:   0.00
  UNIDENT PK FACTOR: 0.000000
  SLICE WIDTH:  10

PAGE 2
  SAMPLE PARAMETERS
  RUN TYPE: A
  SAMPLE ID: DN-S-20-D1
  DIVISOR: 100.0000
  AMT STD: 0.217600
  MLTPLR: 100.0000

PAGE 3
  REPORT INSTRUCTIONS
  WHERE TO REPORT: L
  COPIES: 1
  TITLE: CARBOHYDRATES
  FORMAT: E
  DECIMAL PLACE: 3
  RESULT UNITS: %WETWT
  REPORT UNIDENT PKS: Y
  REPORT INSTRUMENT CONDITIONS: N

PAGE 4
  PLOT INSTRUCTIONS
  PLOT: Y
  ZERO OFFSET:  2
  ANNOTATION
    RETENTION TIME: Y
    PLOT CONTROL: Y
    TIME TICKS: Y
    TIME EVENTS: Y
    PK START/END: Y

PAGE 5
  CHART SPEED
    PAGES OR CM/MIN: C
    INIT VALUE:  1.0

PAGE 6
  PLOT ATTEN
    INIT PLOT ATTEN: 512
```

```
SECTION 2: TIME EVENTS
PAGE 1
   LINE#  TIME  EVENT   VALUE
   1       0.00  PR      50000
   2       0.00  SN        100
   3       0.00  T%        5.0
   4       0.00  WI          4
   5       0.00  II       2.50


SECTION 3: PEAK TABLE
PAGE 1
  STD PK#:  1
  RELATIVE RETEN PK#:  1
  RESOLUTION PK#:  0
  RESOLUTION MINIMUM:   0.0
  FACT%:  25.0
  IDENTIFICATION TIME WINDOWS +/-
  REF
    %: 10
    MIN:   0.00
  NON REF
    %:  5
    MIN:   0.00

PAGE 2
   PK# TIME  NAME         FACTOR    AMOUNT REF
   1    3.78 *RHAMNOSE   1.000000 41.40000 R
   2    5.31 *GLUCOSE    0.963352 43.20000 R
   3    6.02 *SORBITOL   0.478866 25.60000 R
   4    6.82 *FRUCTOSE   0.932840 61.90000 R
   5   10.62 *SUCROSE    1.037920 79.30000 R


SECTION 4: GC INSTRUMENT CONTROL
PAGE 1
  COL TEMP
    ISO/INIT COL TEMP: 140
    INIT HOLD TIME:   0.00
   STEP#   FINAL TEMP   RATE   HOLD TIME
   1           300       20.0       4.00

PAGE 2
  DETECTORS
  DET A TYPE: FID
  DET B TYPE:
   LN#  TIME SIDE ATTN RANGE ZERO
   1     0.00  A   512   11    Y
   2     0.00  B               Y
```

```
PAGE 3
  TEMP/FLOW
  INJ A TEMP: 310
  INJ B TEMP: 310
  ION TEMP: 320
  TCD TEMP:
  TCD FIL TEMP:
  AUX TEMP:
  COL A FLOW: 30
  COL B FLOW: 40

PAGE 4


SECTION 7: POST RUN
PAGE 1
  FILE NAME: DNA
  SAVE INSTRUCTIONS
    TYPE: RAW
    WHERE TO SAVE: L
  TRANSMIT/REPLOT INSTRUCTIONS
    TRANSMIT RAW DATA: N
    REPLOT WITH BASELINES: N
    RAW DATA LOCATION: L
    TRANSMIT REPORT: N

PAGE 2
  METHOD LINKING INSTRUCTIONS
    METHOD:
    LINK CALC RESULTS: N
  PROGRAM EXECUTION
    PROGRAM:
    PARAMETERS:
    RESERVE PRINTER: Y


SECTION 10: NOTE PAD
PAGE 1
   LINE#        VALUE
    1        COL TEMP 140-300 AT 20/MIN
    2        5 UL INJ
    3        DANDELION STEM 1ST INJ 2ND DAY
    4        1ST DERIVATIVE
```

APPENDIX B

RETENTION TIMES, RELATIVE RETENTION TIMES, AND EXPECTED CHEMICAL IONIZATION M/e VALUES OF REFERENCES

RET. TIME	RRT	COMPOUND	NORMAL (M+18)	N-15 (M+18)	DEUT. (M+18)
2.50	0.63	ERYTHROSE	261	262	270
3.40	0.85	ERYTHRITOL	308	---	320
3.50	0.88	DIGITOXOSE	289	290	298
3.70	0.93	2-DEOXY-RIBOSE	275	276	284
3.80	0.95	METHYL-beta-D-XYLOPYRANOSIDE	308	---	317
4.00	1.00	RHAMNOSE	347	348	359
4.10	1.03	RIBOSE	333	334	345
4.40	1.10	XYLOSE	333	334	345
4.80	1.20	RIBITOL	380	---	395
5.10	1.28	2-DEOXY-GLUCOSE	347	348	359
5.50	1.38	METHYL-beta-D-GLUCOPYRANOSIDE	380	---	392
5.60	1.40	GLUCOSE	405	406	420
6.00	1.50	RIBULOSE	393	394	408
6.10	1.53	INOSITOL	450	---	468
6.20	1.55	SORBITOL	452	---	470
6.30	1.58	LINAMARIN	433	---	445
6.50	1.63	N-ACETYL-GLUCOSAMINE	404	405	416
6.70	1.68	GLUCOHEPTOSE	477	478	495
6.80	1.70	LOTAUSTRALIN	447	---	459
7.10	1.78	FRUCTOSE	465	466	483
7.30	1.83	PERSEITOL	524	---	545
8.30	2.08	MANNOHEPTULOSE	537	538	558
9.50	2.38	SALICIN	514	---	529
10.9	2.73	MALTOSE	693	694	717
11.3	2.83	SUCROSE	696	---	720
11.3	2.83	CELLOBIOSE	693	694	717
11.3	2.83	LAMINARABIOSE	693	694	717
11.3	2.83	TREHALOSE	696	---	720
12.5	3.13	GENTIOBIOSE	693	694	717

TABLE 1

REPETITIVE 3 uL INJECTIONS OF THE SAME REFERENCE SOLUTION

Recording Integrator Calculations for a Hypothetical Wet-Weight Percent from the GC Peaks of the Below Sugars[a]

Injection #	D-Glucose	Sorbitol	D-Fructose	Sucrose
1	105	73	116	85
2	105	74	116	87
3	104	73	111	84
4	105	72	115	86
5	107	73	113	87
6	106	74	114	87
7	107	73	116	85
8	106	72	121	84
9	104	72	117	87
x_m	105.4[b]	72.8	115.4	85.8
s_{n-1}	1.1	0.8	2.8	1.3

[a] The chromatographic conditions are described in Appendix A, and these calculations are relative to an internal standard of the PAAN derivative of L-rhamnose

[b] For the last two lines "x_m" is the mean of the above values, and s_{n-1} is the standard deviation of those values.

TABLE 2

TRIPLICATE 3 uL INJECTIONS FROM FIVE PARALLEL DERIVATIZATIONS OF THE SAME REFERENCE SOLUTION

Recording Integrator Calculations for a Hypothetical Wet-Weight Percent from the GC Peaks of the Derivatives of the Below Sugars[a]

Inject. #	Deriv. #	D-Glucose	Sorbitol	D-Fructose	Sucrose
1	R9	105	73	122	85
2	R9	105	74	116	87
3	R9	105	73	108	84
4	R10	110	78	116	86
5	R10	109	75	116	87
6	R10	111	77	111	87
7	R11	111	73	115	85
8	R11	111	72	113	84
9	R11	113	74	114	87
10	R12	105	78	116	82
11	R12	107	81	125	83
12	R12	106	73	117	82
13	R13	107	73	125	85
14	R13	106	72	127	84
15	R13	104	72	126	82
x_m	all	107.7[b]	74.5	117.8	84.7
s_{n-1}	all	2.9	2.7	5.8	1.9

([a] and [b] are the same footnotes as in Table 1)

TABLE 3

INJECTIONS FROM DERIVATIZATIONS FROM THE SAME EXTRACT SOLUTION OF PLANT TISSUE (PETALS OF THE HAWTHORNE, Craategus sp.) WITH VARYING EVAPORATION PROCEDURES BEFORE DERIVATIZATION

Recording Integrator Calculation for the Wet-Weight Percent of the Below Sugar in Terms of the Original Tissue[a]

Evap.[b]	Ref.[c]	D-Glucose	Sorbitol	D-Fructose	Sucrose
A (-)	1.34	0.506	0.91	1.30	0.161
B (85)	1.42	0.638	1.00	1.15	0.160
C (70)	1.44	0.715	1.00	1.22	0.166
D (50)	1.39	0.641	1.00	1.13	0.166
C (70)[d]	1.45	0.713	0.954	1.19	0.166
C (70)[d]	1.48	0.698	0.961	1.209	0.167
C (70)[d]	1.43	0.718	0.965	1.232	0.163
C (70)[d]	1.46	0.715	0.957	1.204	0.160
x_m[e]	-	0.711	0.959	1.208	0.164
s_{n-1}[e]	-	0.0089	0.0048	0.018	0.0032

[a] Calculated relative to the peak of the L-rhamnose internal standard.
[b] Evaporation conditions, refer to the text for specific conditions. The number in parenthesis is the block temperature in °C.
[c] Reference area count (in million) on the Vista system
[d] The average of two chromatograms from a single derivatization.
[e] The mean and the standard deviation are calculated for the last four "condition C" determinations.

TABLE 4

LINEARITY OF THE FID DETECTOR RESPONSE WITH A CONSTANT INTERNAL STANDARD AND VARYING AMOUNTS OF COMPARISON SUGARS

Detector Response verses Actual Amount Injected in Terms of ugm.

Dilution	D-Glucose		Sorbitol		D-Fructose		Sucrose	
Ratio[a]	Inj[b]	Res[b]	Inj	Res	Inj	Res	Inj	Res
0.25	1.95	2.11[c]	1.28	1.40	1.78	3.32	4.09	4.08
0.35	2.80	2.95	1.82	1.96	3.48	4.66	5.68	5.71
0.50	4.24	4.21	3.62	2.80	5.60	6.65	8.22	8.15
0.75	6.34	6.32	4.86	4.20	9.30	9.98	12.41	12.23
1.00	8.42[d]	8.42	6.07	5.60	12.38	13.30	16.30[d]	16.30
1.25	11.82	10.53	7.77	7.00	17.35	16.63	21.57	20.38
1.50	12.82	12.63	8.87	8.40	19.95[d]	19.95	24.54	24.45
2.00	17.18	16.81	11.20[d]	11.20	27.24	26.60	32.92	32.63
2.50	21.59	21.09	13.76	14.00	33.22	33.25	41.63	40.84
3.00	24.91	25.29	15.43	16.80	36.91	39.90	48.51	48.92
3.50	29.32	29.53	17.62	19.60	42.22	46.55	58.01	57.12

[a] The dilution ratio describes the amount of L-rhamnose added, relative to the other sugars, and is defined in the text.
[b] The term "Inj" refers to the amount of sugar derivative (based on the weight of the original sugar) used in each injection. "Res" referss to the detector response in units which are normalized to the amount of injected sugars.
[c] Each response value represents the average of three chromatographic runs.
[d] This is the specific value which was normalized for the detector response.

TABLE 5

LINEARITY OF THE FID DETECTOR RESPONSE WITH BOTH A CONSTANT INTERNAL STANDARD LEVEL AND A CONSTANT AMOUNT OF SUGAR EXTRACT FROM A PLANT TISSUE (PETALS OF THE HAWTHORNE, Crategus sp.), BUT WITH VARYING ADDITIONAL AMOUNTS OF ADDED SUGARS

Increased Percentage Wet-Weight of Saccharide Calculated by the Recording Integrator (based upon GC Response) Compared to the Amount of Saccharide that would have been Added to the Plant Tissue due to the Added Saccharide Volume

Volume	D-Glucose		Sorbitol		D-Fructose		Sucrose	
Added[a]	Inj[b]	Rec[c]	Inj[b]	Rec[c]	Inj[b]	Rec[c]	Inj[b]	Rec[c]
000	0.00	0.71[d]	0.00	0.96[d]	0.00	1.21[d]	0.00	0.16[d]
200	0.39	0.40[e]	0.36	0.35[e]	0.54	0.54[e]	0.12	0.13[e]
400	0.78	0.79	0.72	0.73	1.08	1.10	0.24	0.24
600	1.17	1.17	1.08	1.05	1.62	1.60	0.36	0.35
800	1.56	1.55	1.40	1.35	2.16	2.21	0.48	0.46

[a] The volume of the additional saccharide solution (in uL).
[b] "Inj" refers to the calculated amount of extra percent wet-weight sugar which would have been in one gram of plant tissue extract, based upon both the volume and concentration of the added saccharide solution.
[c] "Rec" refers to the increase (over the original wet-weight percentage of that saccharide) in wet-weight percentage as calculated by the recording integrator.
[d] The wet-weight percentage in each extract from the original "un-spiked" Hawthorne petals.
[e] This value, and all values in the below column, refer to the difference between the percent wet-weight value calculated by the recording integrator and the above "d" value for a "non-spiked" solution. Each of these wet-weight percentage values is the mean of the values from two chromatograms each of two derivatives from each of two spiked solutions. See the pentultimate line in Table 3 for the data for the non-spiked solutions, which is the same as the above first line of data.

TABLE 6

AGREEMENT OF THE ANALYSES OF FREE SUGARS IN THE EXTRACTS OF PLANT TISSUES OF SIMILAR SOURCE (PETALS OF THE WILD ROSE, TAKEN AT THE SAME TIME) BUT FROM DIFFERENT INDIVIDUAL PLANTS WHICH WERE SEPARATED FROM EACH OTHER BY FIVE METER INTERVALS

Wet-weight Sugar Percentages, Based upon the FID Detector Response from Gas Liquid Chromatography [a]

Plant[b] #	Derivative[c] #	Injection #	D-Glucose	Sorbitol	D-Fructose	Sucrose
1	1	1	1.86	0.11	1.67	0.20
1	1	2	1.84	0.10	1.73	0.20
1	2	1	1.79	0.11	1.60	0.19
1	2	2	1.82	0.11	1.70	0.20
1	Avg	-	1.83[d]	0.11[e]	1.67	0.20
2	1	1	1.76	0.10	1.62	0.21
2	1	2	1.78	0.10	1.65	0.21
2	2	1	1.81	0.10	1.55	0.21
2	2	2	1.80	0.10	1.56	0.20
2	Avg	-	1.79	0.10[e]	1.60	0.21
3	1	1	1.81	0.11	1.66	0.22
3	1	2	1.83	0.11	1.67	0.21
3	2	1	1.81	0.11	1.66	0.22
3	2	2	1.84	0.11	1.74	0.22
3	Avg	-	1.82	0.11[e]	1.67	0.22

[a] The tissue samples were prepared and analyzed as described in Appendix A.
[b] See the text for the location of the specific plants.
[c] Three flowers were taken from each plant and the petals commingled into a single extract. Each extract was derivatized once, and each derivative was chromatographed twice.
[d] The underlined values represent the mean of the above four values.
[e] These data are shown as calculated, but actually represent the side-product of the L-rhamnose internal standard. NO sorbitol was identified in these extracts.

TABLE 7

DANDELION (Taraxacum officinale): A TRANSVERSE STUDY (IN TERMS OF TIME) OF THE WET-WEIGHT PERCENTAGES OF THE MAJOR FREE SUGARS PRESENT IN THE TISSUES OF THE STEM

Wet-weight Sugar Percentages, Based upon the FID detector Response from Gas Liquid Chromatography[a]

Day[b]	Sample Area[c]	D-Glucose	Sorbitol	D-Fructose	Sucrose
1	1	0.83[d]	0.00[e]	0.05	0.22
1	2	1.33	0.00	0.06	0.28
1	Avg	1.08[f]	0.00	0.06	0.25
2	1	1.44	0.01	0.25	0.28
2	2	1.64	0.00	0.13	0.31
2	Avg	1.54	0.00	0.19	0.30
4[g]	1	1.14	0.01	0.16	0.26
4	2	1.27	0.01	0.26	0.44
4	Avg	1.21	0.01	0.21	0.35
5	1	1.09	0.00	0.12	0.26
5	2	1.10	0.01	0.10	0.28
5	Avg	1.10	0.00	0.11	0.27

[a] The tissue samples were prepared and analyzed as defined in Appendix A.
[b] Sequential days, starting June 27, 1986.
[c] The sample areas are defined in the text.
[d] The average of two chromatograms, one each from duplicate derivatizations of a single extract from three plants.
[e] All values in this column were subtracted from 0.023, the average value of the side-product of L-rhamnose.
[f] The underlined values are the average of the above two determinations (the average of the two areas sampled).
[g] No samples were collected on the third day.

TABLE 8

THE FID DETECTOR RESPONSE FOR THE PEAK AREAS OF CHROMATOGRAMS FROM DERIVATIZATIONS WITH DIFFERING REACTION TIMES FOR THE FIRST (OXIME-FORMATION) STEP

FID Response of the GC Peaks as Area Counts for the Derivatives Resulting from the Below Starting Sugars[a]

Time (min)[b]	L-Rhamnose	D-Glucose	Sorbitol	D-Fructose	Sucrose
10	1.10[c]	1.22	1.54	1.19	2.84
15	1.10	1.24	1.55	1.15	2.85
20[d]	1.07	1.22	1.56	1.16	2.80
25	1.05	1.19	1.50	1.16	2.77

[a] See the text for the preparation of these derivatives.
[b] The first step (oxime-formation) reaction time.
[c] Area counts (in millions) from the Varian Vista system. The reactions were done in duplicate for each condition, with each reaction extract chromatographed twice. Therefore, each value in this table is the average of four chromatograms.
[d] This line corresponds to the "standard" reaction conditions in Appendix A, and is the same data in line 3 of Table 9.

TABLE 9

THE FID DETECTOR RESPONSE FOR THE PEAK AREAS OF CHROMATOGRAMS FROM DERIVATIZATIONS WITH DIFFERING REACTION TIMES FOR THE SECOND (ACETYLATION) STEP

FID Response of the GC Peaks as Area Counts for the Derivatives Resulting from the Below Starting Sugars[a]

Time (min)[b]	L-Rhamnose	D-Glucose	Sorbitol	D-Fructose	Sucrose
20	0.98[c]	1.12	1.49	1.13	2.67
25	0.97	1.11	1.44	1.13	2.59
30[d]	1.07	1.22	1.56	1.16	2.80
35	1.09	1.24	1.57	1.18	2.85

[a] See the text for the preparation of these derivatives.
[b] The second step (acetylation) reaction time.
[c] Area counts (in millions) from the Varian Vista system. The reactions were done in duplicate for each condition, with each reaction extract chromatographed twice. Therefore, each value in this table is the average of four chromatograms.
[d] This line corresponds to the "standard" reaction conditions in Appendix A, and is the same data in line 3 of Table 8.

TABLE 10

COMPARISON OF THE PEAK AREAS OF CHROMATOGRAMS RESULTING FROM A UNIFORM AMOUNT OF STARTING SACCHARIDE WHICH WAS DERIVATIZED BY ALTERNATIVE PROCEDURES

FID Response of the GC Peaks as Area Counts for the Derivatives Resulting from the Below Starting Sugars[a]

Method (by the catalyst)	L-Rhamnose	D-Glucose	Sorbitol	D-Fructose	Sucrose
Pyridine [b]	1.05[c]	1.19	1.54	1.18	2.82
NMIM[d]	0.63	0.78	1.16	0.40	2.37
DMAP[e]	1.04	1.23	1.50	1.15	2.87

[a] Area count (in millions) form the Varian Vista system when 4 uL samples were injected with a Chaney adapter.
[b] The method described in Appendix A.
[c] Values established from the average of duplicate injections of duplicate reactions for each reaction condition (an average of four values).
[d] The method of Chen and McGinnis (which employed N-methyl-imidazole) as described in reference 36.
[e] The method of Guerrant and Moss (which employed dimethylaminopyridine) as described in reference 38.

TABLE 11

COMPARISON OF THE PEAK AREAS OF CHROMATOGRAMS RESULTING FROM UNIFORM AMOUNTS OF STARTING SACCHARIDES WITH THE ACETYLATION STEP CATALYZED BY TEN PERCENT N-METHYL-IMIDAZOLE UNDER VARYING REACTION CONDITIONS

FID Response of the GC Peaks as Area Counts[a] for the Derivatives Resulting from the Below Starting Saccharides

Temperature (°C)	Time (min)	L-Rhamnose	D-Glucose	Sorbitol	D-Fructose	Sucrose
20	5	0.68[b]	0.82	1.05	0.97	2.38
70	10	0.98	1.01	1.37	1.01	2.58
70	20	1.07	1.11	1.45	1.13	2.75

[a] Area counts (in millions) from the Varian Vista system, when 4 uL samples were injected with a Chaney adapter. Due to identical amounts of starting materials, and identical extraction and chromatographic procedures, the data in this table may be directly compared to those in Tables 8, 9, 10, and 12.

[b] These values are the average of duplicate derivatizations for each reaction condition, and the duplicate injection of the extract of each derivatization.

TABLE 12

COMPARISON OF THE PEAK AREAS OF CHROMATOGRAMS RESULTING FROM UNIFORM AMOUNTS OF STARTING SACCHARIDES WITH THE ACETYLATION STEP, OF TEN MINUTES DURATION, CATALYZED BY VARYING PERCENTAGES OF N-METHYL-IMIDAZOLE

FID Response of the GC Peaks as Area Counts[a] for the Derivatives Resulting from the Below Starting Saccharides

NMIM Added (uL)	NMIM to Pyridine Ratio	L-Rhamnose	D-Glucose	Sorbitol	D-Fructose	Sucrose
25	1:8	0.98[b]	1.11	1.35	1.13	2.59
50	1:4	0.91	0.99	1.24	0.89	2.37
75	1:3	0.96	0.98	1.25	0.78	2.39
100	1:2	0.95	0.98	1.34	0.56	2.48
150	1:1.3	0.83	0.79	1.13	0.19	2.48

[a] Area counts (in millions) from the Varian Vista system, when 4 uL samples were injected with a Chaney adapter. Due to identical amounts of starting materials, and identical extraction and chromatographic procedures, the data in this table may be directly compared to those in Tables 8 through 11.

[b] Data from single injections of a single derivative for each reaction condition.

TABLE 13

CANADA THISTLE (Cirsium arvense): A TRANSVERSE STUDY (IN TERMS OF TIME) OF THE WET-WEIGHT PERCENTAGES OF THE MAJOR FREE SUGARS PRESENT IN THE TISSUES OF THE STEM

Wet-weight Sugar Percentages, Based upon the FID detector Response from Gas Liquid Chromatography[a]

Day[b]	Sample Area[c]	D-Glucose	Sorbitol	D-Fructose	Sucrose
1	1	0.16[d]	0.00[e]	0.29	0.37
1	2	0.11	0.00	0.29	0.44
1	Avg	0.14[f]	0.00	0.29	0.45
2	1	0.03	0.00	0.12	0.43
2	2	0.04	0.00	0.24	0.46
2	Avg	0.04	0.00	0.18	0.45
4[g]	1	0.07	0.00	0.15	0.76
4	2	0.08	0.00	0.19	0.84
4	Avg	0.07	0.00	0.17	0.80
5	1	0.05	0.00	0.12	0.62
5	2	0.13	0.00	0.21	0.83
5	Avg	0.09	0.00	0.16	0.72

[a] The tissue samples were prepared and analyzed as defined in Appendix A.
[b] Sequential days, starting June 27, 1986.
[c] The sample areas are defined in the text.
[d] The average of two chromatograms, one each from duplicate derivatizations of a single extract from three plants.
[e] All values in this column were subtracted from 0.028, the average value of the side-product of L-rhamnose.
[f] The underlined values are the average of the above two determinations (the average of the two areas sampled).
[g] No samples were collected on the third day.

TABLE 14

FIELD BINDWEED (Convolvulus arvensis): A TRANSVERSE STUDY (IN TERMS OF TIME) OF THE WET-WEIGHT PERCENTAGES OF THE MAJOR FREE SUGARS PRESENT IN THE TISSUES OF THE STEM

Wet-weight Sugar Percentages, Based upon the FID detector Response from Gas Liquid Chromatography[a]

Day[b]	Sample Area[c]	D-Glucose	Sorbitol	D-Fructose	Sucrose
1	1	0.30[d]	0.00[e]	0.00	0.34
1	2	0.11	0.00	0.00	0.33
1	Avg	0.20[f]	0.00	0.00	0.34
2	1	0.22	0.00	0.00	0.42
2	2	0.12	0.00	0.00	0.31
2	Avg	0.17	0.00	0.00	0.37
4[g]	1	0.21	0.00	0.00	0.59
4	2	0.21	0.00	0.00	0.67
4	Avg	0.21	0.00	0.00	0.63
5	1	0.42	0.00	0.00	0.59
5	2	0.56	0.00	0.00	0.72
5	Avg	0.49	0.00	0.00	0.66

[a] The tissue samples were prepared and analyzed as defined in Appendix A.
[b] Sequential days, starting June 27, 1986.
[c] The sample areas are defined in the text.
[d] The average of two chromatograms, one each from duplicate derivatizations of a single extract from three plants.
[e] All values in this column were subtracted from 0.028, the average value of the side-product of L-rhamnose.
[f] The underlined values are the average of the above two determinations (the average of the two areas sampled).
[g] No samples were collected on the third day.

TABLE 15

THE ELECTRON IMPACT-MASS SPECTRUM FRAGMENTATION IONS OF THE PAAN/PAKO DERIVATIVES OF DISACCHARIDES

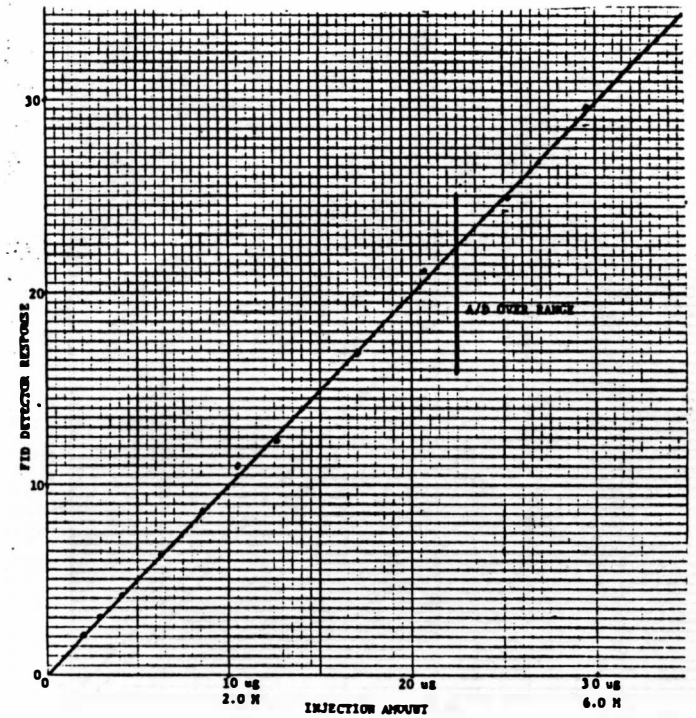
Sucrose	Trehalose	Maltose	Gentiobiose	Laminarabiose
103(5,0,4 & 7)	103(5,0,4 & 7)	103(20,0,4 & 7)	103(15,0,4 & 7)	103(22,0,4 & 7)
109(50,0,0)	109(50,0,0)	109(30,0,0)	109(25,0,0)	109(50,0,0)
----	----	112(45,0,3)	112(25,0,3)	112(35,0,3)
115(10,0,3 & 4)	115(15,0,3 & 4)	115(25,0,3 & 4)	115(25,0,3 & 4)	115(30,0,3 & 4)
127(15,0,1 & 2)	127(15,0,1 & 2)	127(20,0,1 & 2)	127(10,0,1 & 2)	127(20,0,1 & 2)
135(5,0,0)	135(5,0,0)	135(60,0,0)	135(15,0,0)	135(25,0,0)
139(5,0,3)	139(10,0,3)	139(15,0,3)	139(10,0,3)	139(15,0,3)
----	----	140(35,0,3)	140(20,0,3)	140(30,0,3)
----	----	141(15,0,3)	141(10,0,3)	141(15,0,3)
----	----	144(10,0,6)	144(5,0,6)	144(10,0,6)
145(5,0,9)	145(10,0,9)	145(50,0,6 & 9)	145(25,0,6 & 9)	145(45,0,6 & 9)
157(5,0,6)	157(5,0,6)	157(50,0,6)	157(30,0,6)	157(35,0,6)
169(100,0,3 & 4)	169(100,0,3 & 4)	169(100,0,3 & 4)	169(50,0,3 & 4)	169(100,0,3 & 4)
187(5,0,10)	187(5,0,10)	----	----	----
197(5,0,0)	197(2,0,0)	197(20,0,0)	197(15,0,0)	197(20,0,0)
200(1,0,7)	200(2,0,7)	200(25,0,7)	200(15,0,7)	200(20,0,7)
211(70,0,6)	211(5,0,6)	211(10,0,6)	211(10,0,6)	211(10,0,6)
----	----	217(2,0,4)	217(5,0,4)	217(15,0,4)
----	242(5,0,9)	242(30,0,9)	242(10,0,9)	242(10,0,9)
----	243(2,0,9)	243(20,0,9)	243(10,0,9)	243(10,0,9)
271(5,0,9)	271(10,0,9)	----	----	----
----	----	314(70,1,9)	314(35,1,9)	314(55,1,9)
----	----	328(70,1,12)	328(100,1,12)	328(20,1,12)
331(60,0,12)	331(35,0,12)	331(55,0,12)	331(20,0,12)	331(30,0,12)

TABLE 16

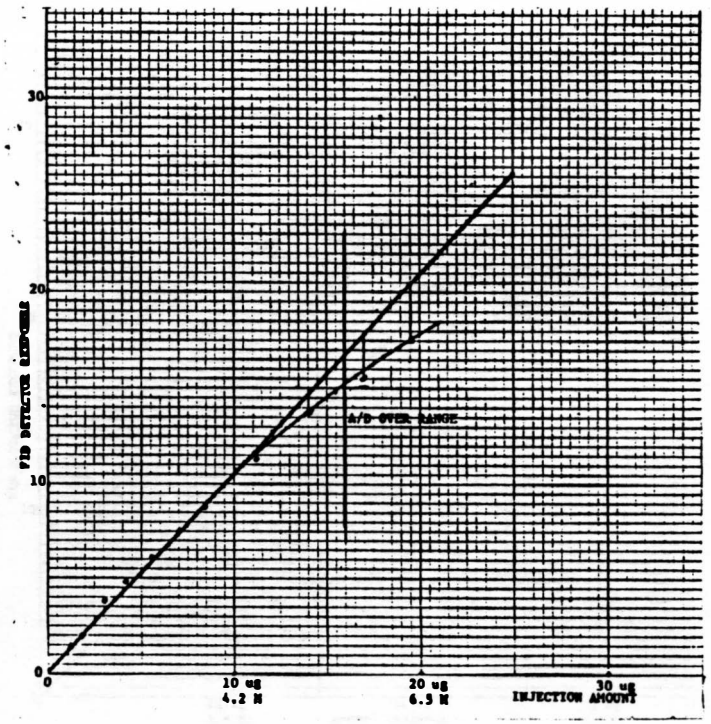
THE ELECTRON IMPACT-MASS SPECTRUM FRAGMENTATION IONS OF THE PAAN/PAKO DERIVATIVES OF COMPLEX GLYCOSIDES

Me-beta-D-Xylo-pyranoside	Me-beta-D-Gluco-pyranoside	Linamarin	Lotaustralin	Salicin
102(25,0,4)	102(95,0,4)	102(70,0,4)	102(70,0,4)	----
103(25,0.7)	103(90,0,7)	103(70,0,7)	103(50,0,7)	----
----	----	----	----	106(15,0,0)
----	----	----	----	107(15,0,0)
----	109(15,0,0)	109(25,0,0)	109(30,0,0)	109(80,0,0)
----	112(90,0,3)	112(90,0,3)	112(80,0,3)	----
115(40,0,3 & 4)	115(100,0,3 & 4)	115(100,0,3 & 4)	115(100,0,3 & 4)	115(10,0,3 & 4)
128(100,0,4)	----	----	----	----
----	----	----	----	135(5,0,0)
139(10,0,3)	139(15,0,3)	139(35,0,3)	139(35,0,3)	139(10,0,3)
----	140(60,0,3)	140(70,0,3)	140(50,0,3)	----
----	141(60,0,3)	141(45,0,3)	141(40,0,3)	----
----	144(55,0,6)	144(65,0,6)	144(45,0,6)	----
145(10,0,9)	145(95,0,6 & 9)	145(85,0,6 & 9)	145(65,0,6 & 9)	145(10,0,6 & 9)
157(40,0,6)	157(95,0,6)	157(95,0,6)	157(85,0,6)	----
----	169(50,0,3)	169(35,0,3)	169(45,0,3)	169(100,0,3)
170(50,0,6)	----	----	----	----
----	----	180(20,0,1)	----	----
----	182(15,0,6)	182(10,0,6)	182(10,0,6)	----
----	200(50,0,7)	200(35,0,7)	200(30,0,7)	----
----	211(5,0,6)	211(10,0,6)	211(5,0,6)	211(5,0,6)
----	----	222(10,0,3)	----	----
----	229(5,0,6)	----	----	229(5,0,6)
----	----	235(10,0,3)	----	----
----	242(15,0,9)	242(15,0,9)	242(10,0,9)	----
----	243(30,0,9)	243(25,0,9)	243(15,0,9)	----
----	----	253(10,0,4)	----	----
259(5,0,9)	259(5,0,9)	259(5,0,9)	259(2,0,9)	259(2,0,9)
	----	----	----	271(5,0,9)
	289(5,0,9)	----	----	----
	331(10,0,12)	331(15,0,12)	331(5,0,12)	331(25,0,12)
		342(5,0,9)	----	
			347(2,0,12)	
			356(2,0,9)	

Example: 103(100^{a},0^{b},4^{c}). The first value in parenthesis, 'a' is the relative ion intensity. The second value in parenthesis, 'b' is the mass increase for the N-15 derivative. The value in parenthesis, 'c' is the mass increase for the Ac-d_3 derivative.

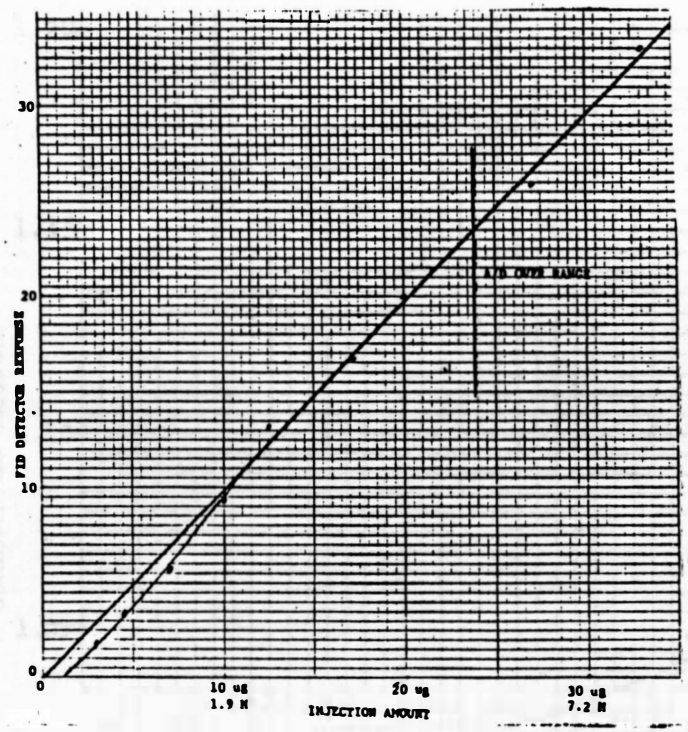


D-Glucose

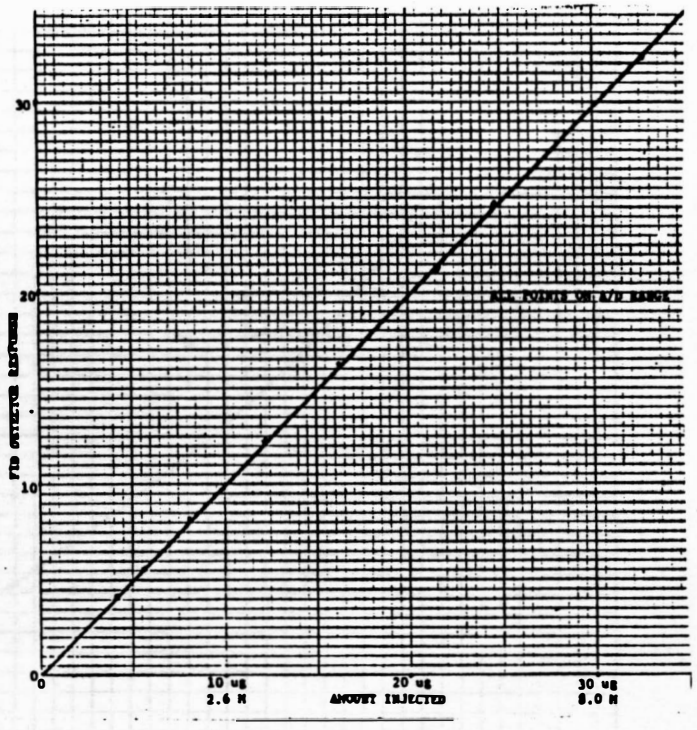


Sorbitol

Figure 1. The FID detector response of the Varian Vista system vs. micrograms of injected saccharide (based upon pre-derivatization weights). The abscissa also indicates the area counts in millions of the Varian Vista system which correspond to these injected amounts. See Table 4 for the original data for these plots.



D-Fructose



Sucrose

Figure 2. The FID detector response of the Varian Vista system vs. micrograms of injected saccharide (based upon pre-derivatization weights). The abscissa also indicates the area counts in millions of the Varian Vista system which correspond to these injection amounts. See Table 4 for the original data for these plots.

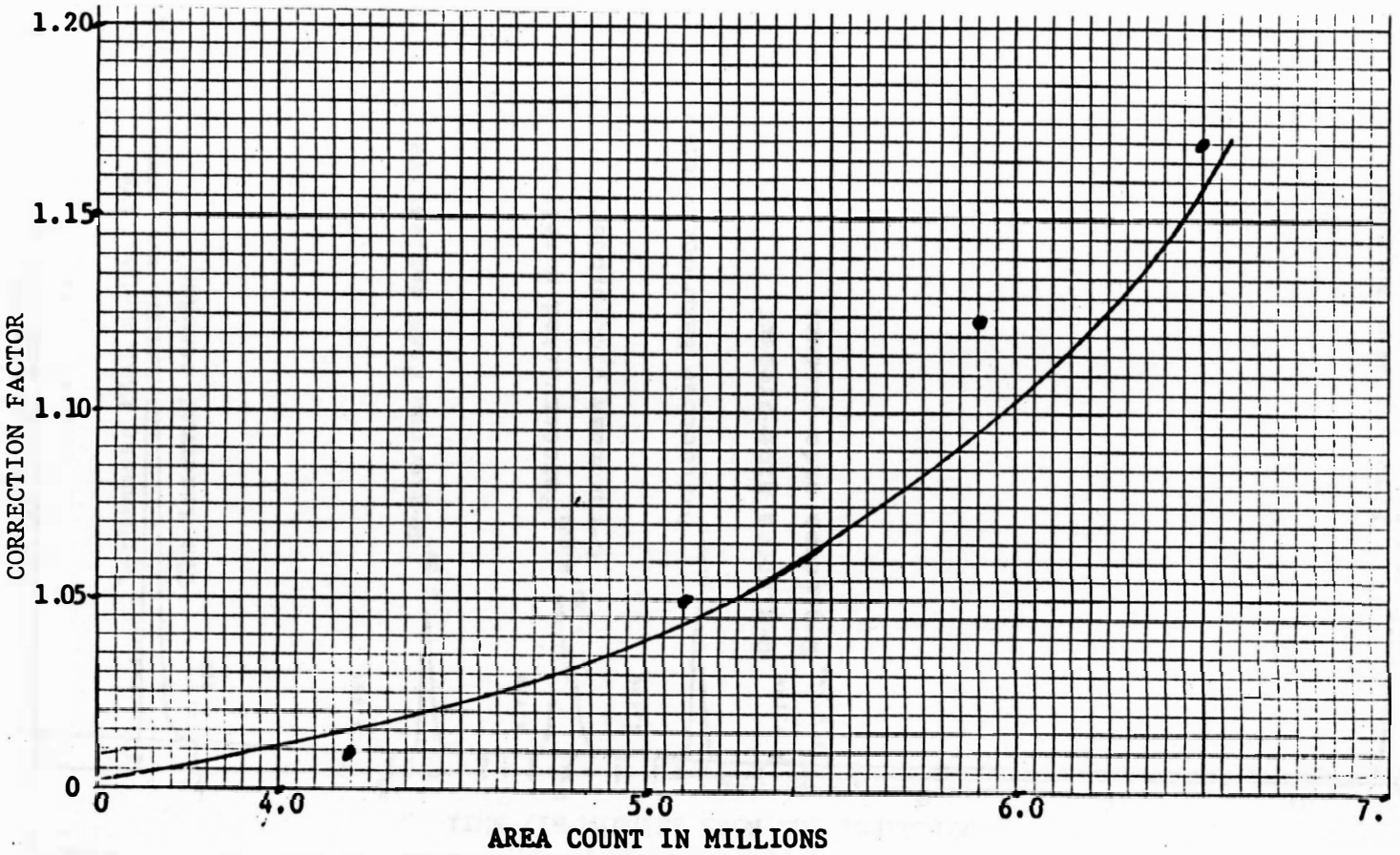


Figure 3. A correction-factor plot for the quantitation of sorbitol in the region beyond chromatographic linearity. First establish the area count of the sorbitol peak, then use the plot to establish the factor, finally multiply this factor times the reported sorbitol.

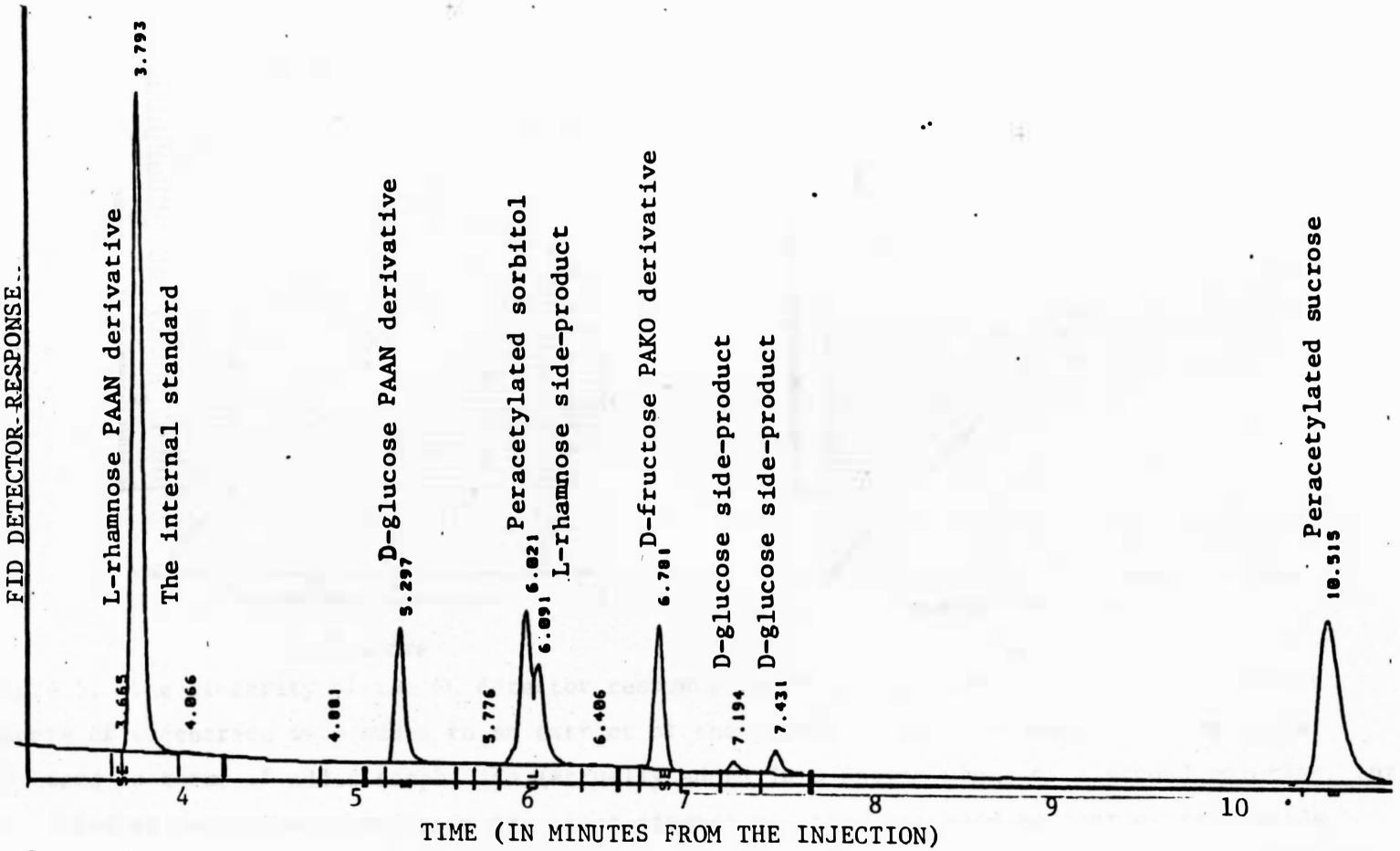
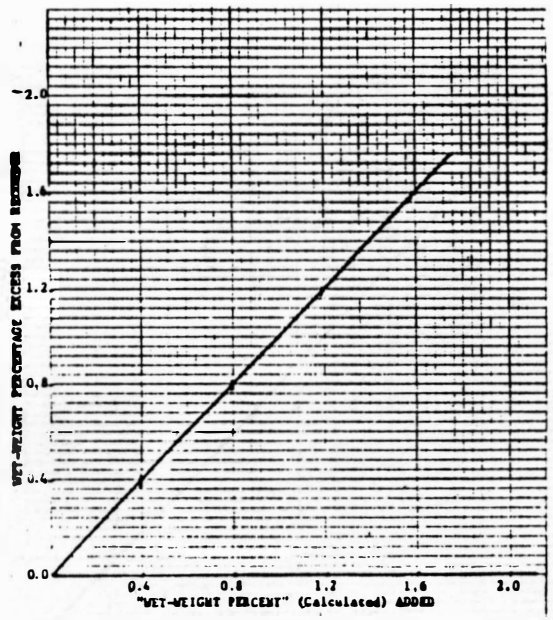
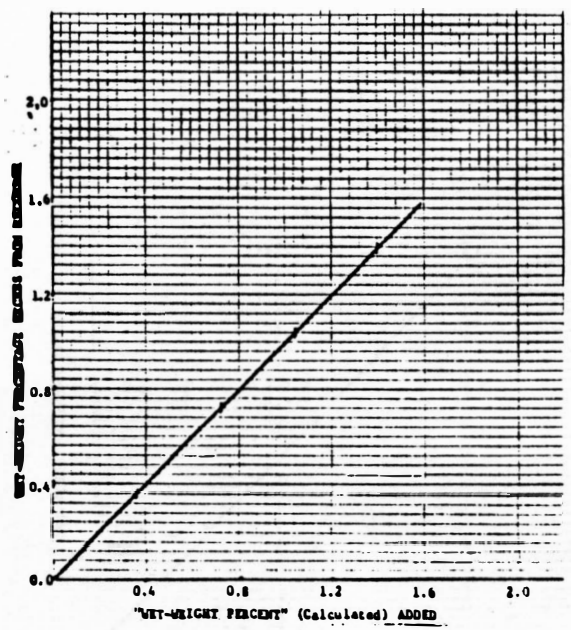


Figure 4. An example chromatogram (at the specific dilution ratio of 0.25) for the study of the linearity of the detector response -- see Table 4 for a summary of the data. These chromatographic conditions are described in Appendix A.

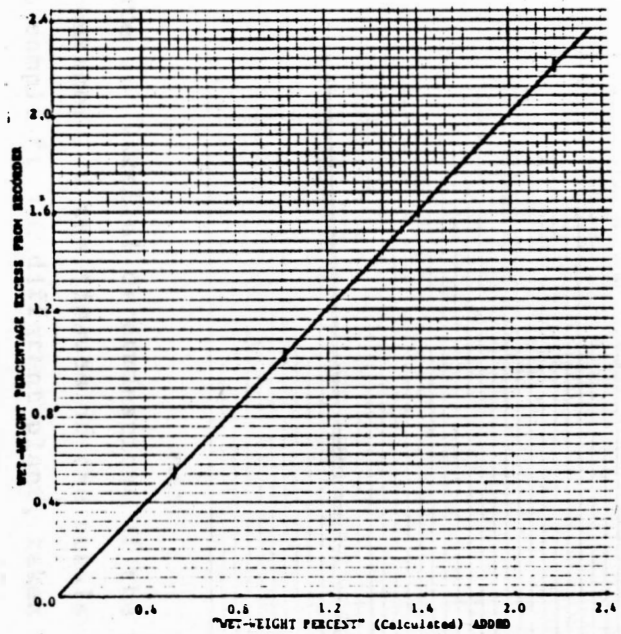


D-Glucose

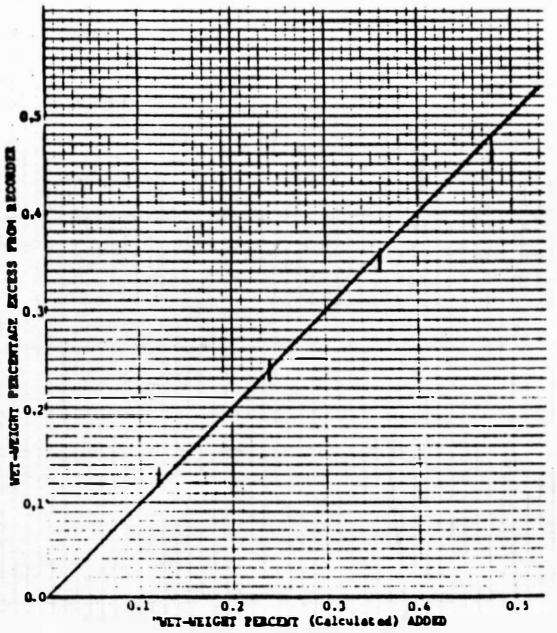


Sorbitol

Figure 5. The linearity of the GC detector response for D-glucose and sorbitol, when various amounts of saccharide were added to an extract of the petals of the Hawthorne. The data are expressed in terms of added saccharide (actually added in a known volume of a second solution, but calculated as percent-saccharide in the plant tissue) vs. the increased percent of saccharide reported by the computing integrator.



D-Fructose



Sucrose

Figure 6. The linearity of the GC detector response for D-fructose and sucrose, when various known amounts of saccharide were added to an extract of the petals of the Hawthorne. The data are expressed in terms of added saccharide (actually added in a known volume of a second solution, but calculated as saccharide percent in the plant tissue) vs. the increased percent of saccharide reported by the computing integrator.

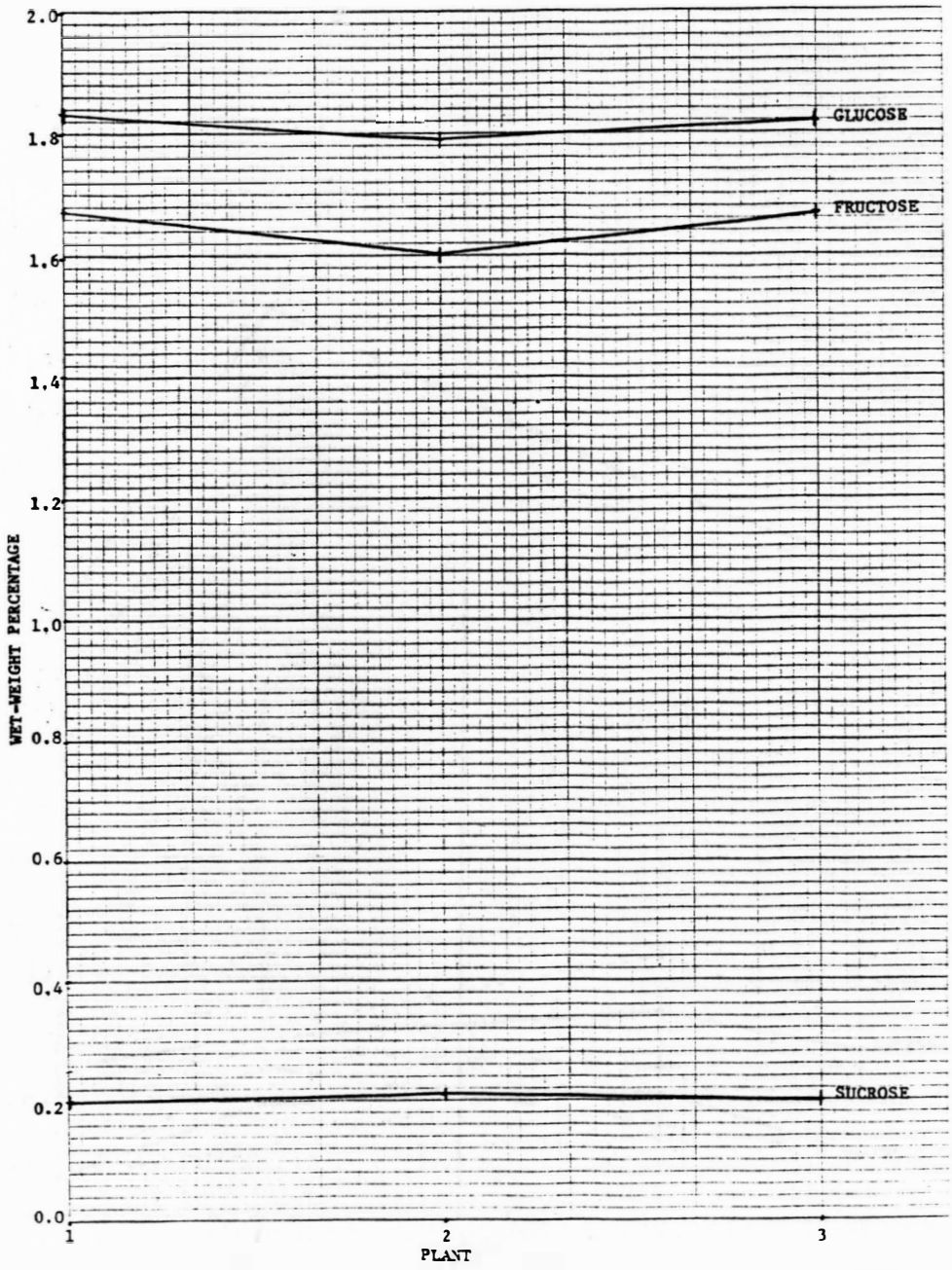


Figure 7. Parallel determinations of the wet-weight saccharide percentages for the tissues of the petals of the wild rose. Each sample from a different plant, taken at the same time.

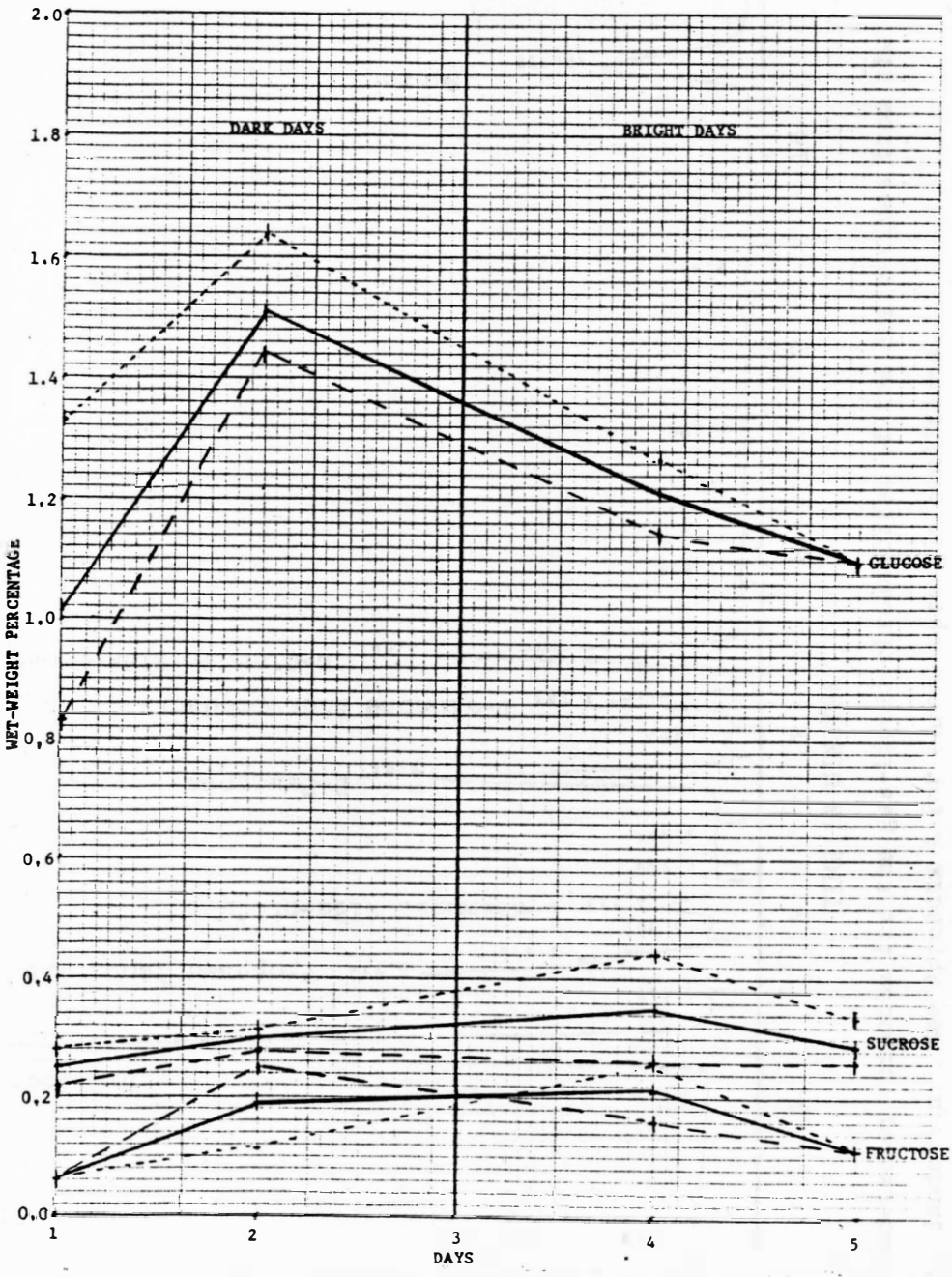


Figure 8. Wet-weight sugar percentages in the stem tissues dandelions as a function of time (in days). The dotted and dashed lines refer to specific growing areas, the solid line to the average value from the two areas.

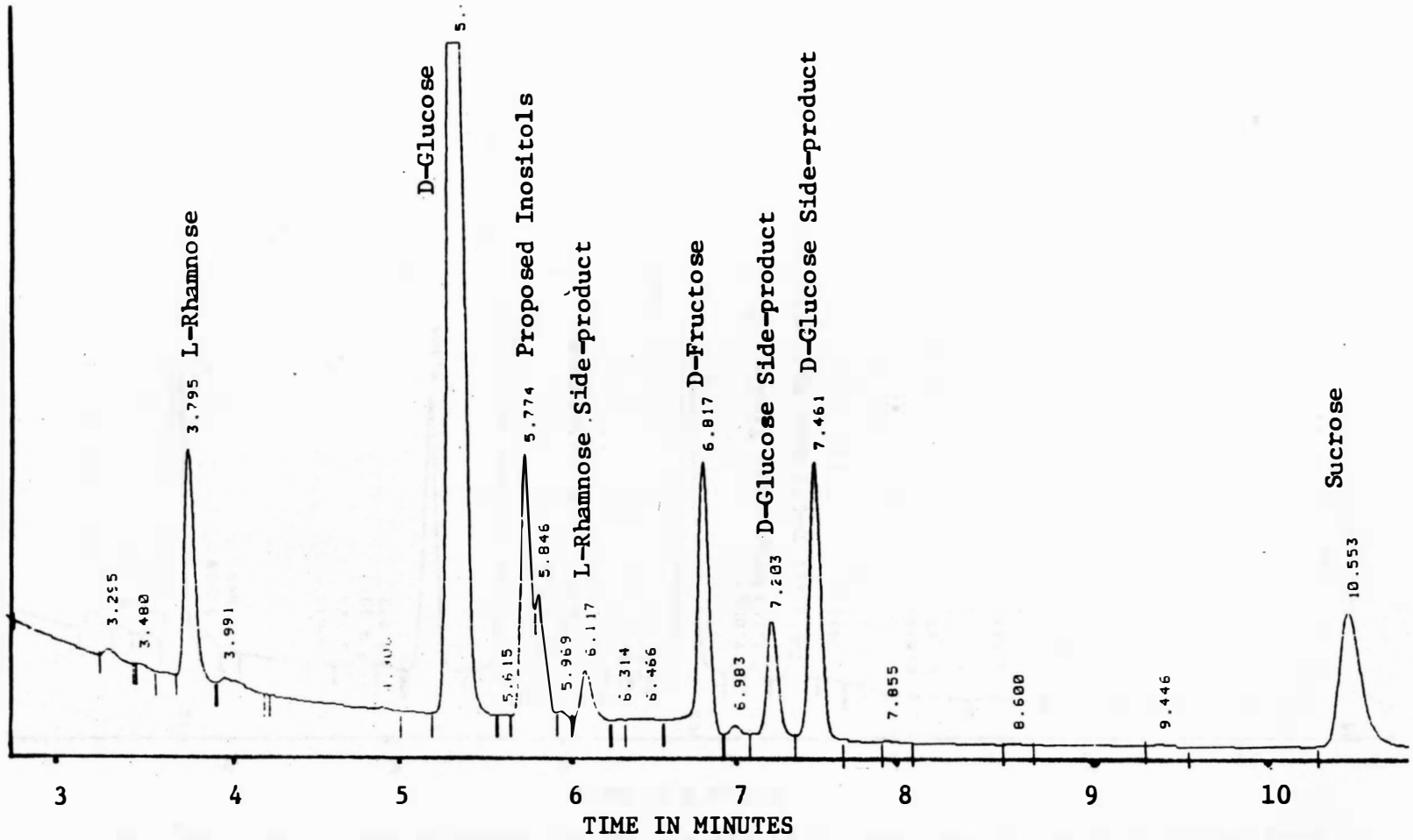


Figure 9. The FID-detector response for the gas-liquid chromatogram of the derivatives from the stem-tissue of the dandelion. See Appendix A for chromatographic conditions.

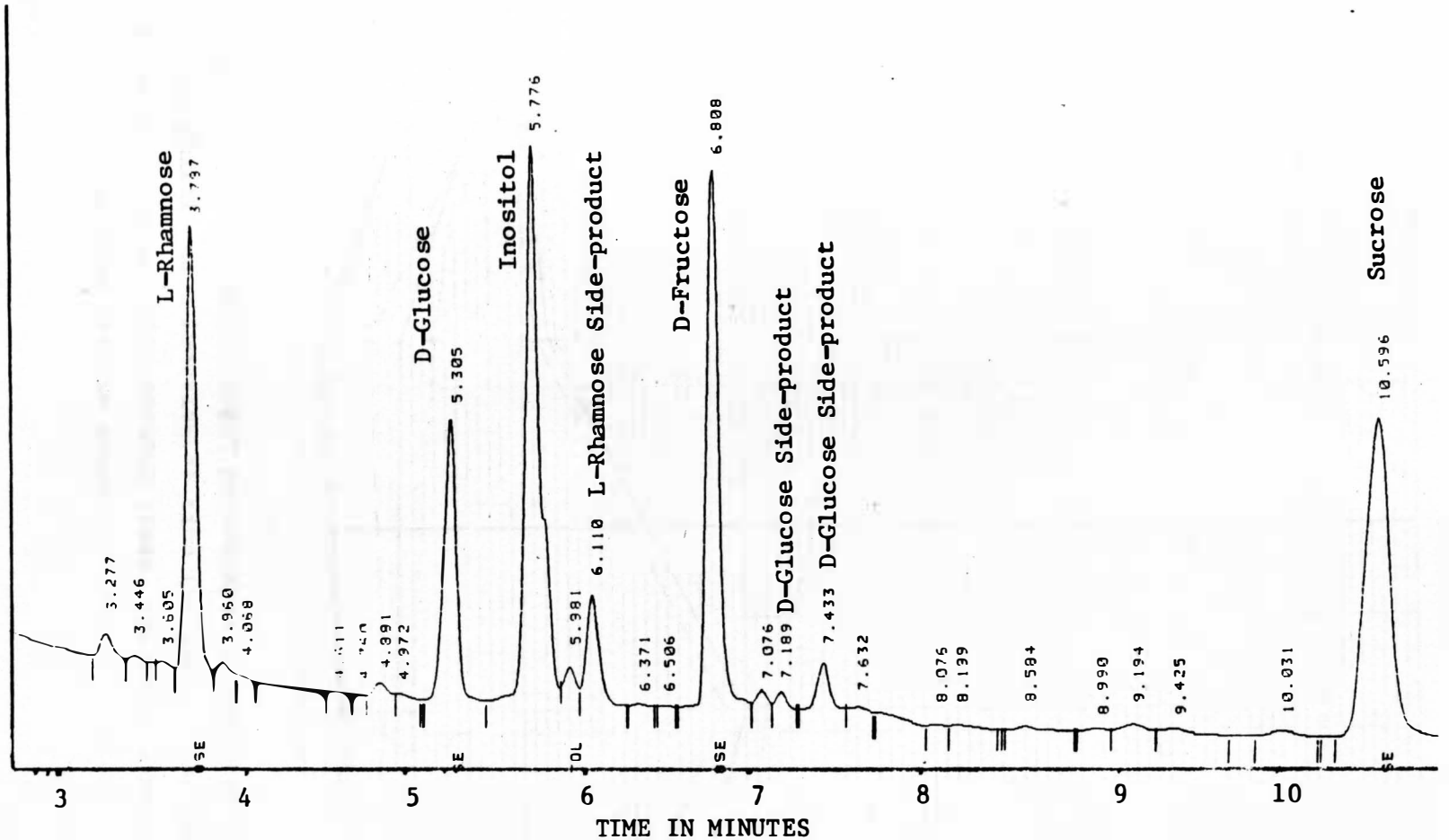


Figure 10. The FID-detector response for the gas-liquid chromatogram of the derivatives from the stem-tissue of the Canada thistle. See Appendix A for chromatographic conditions.

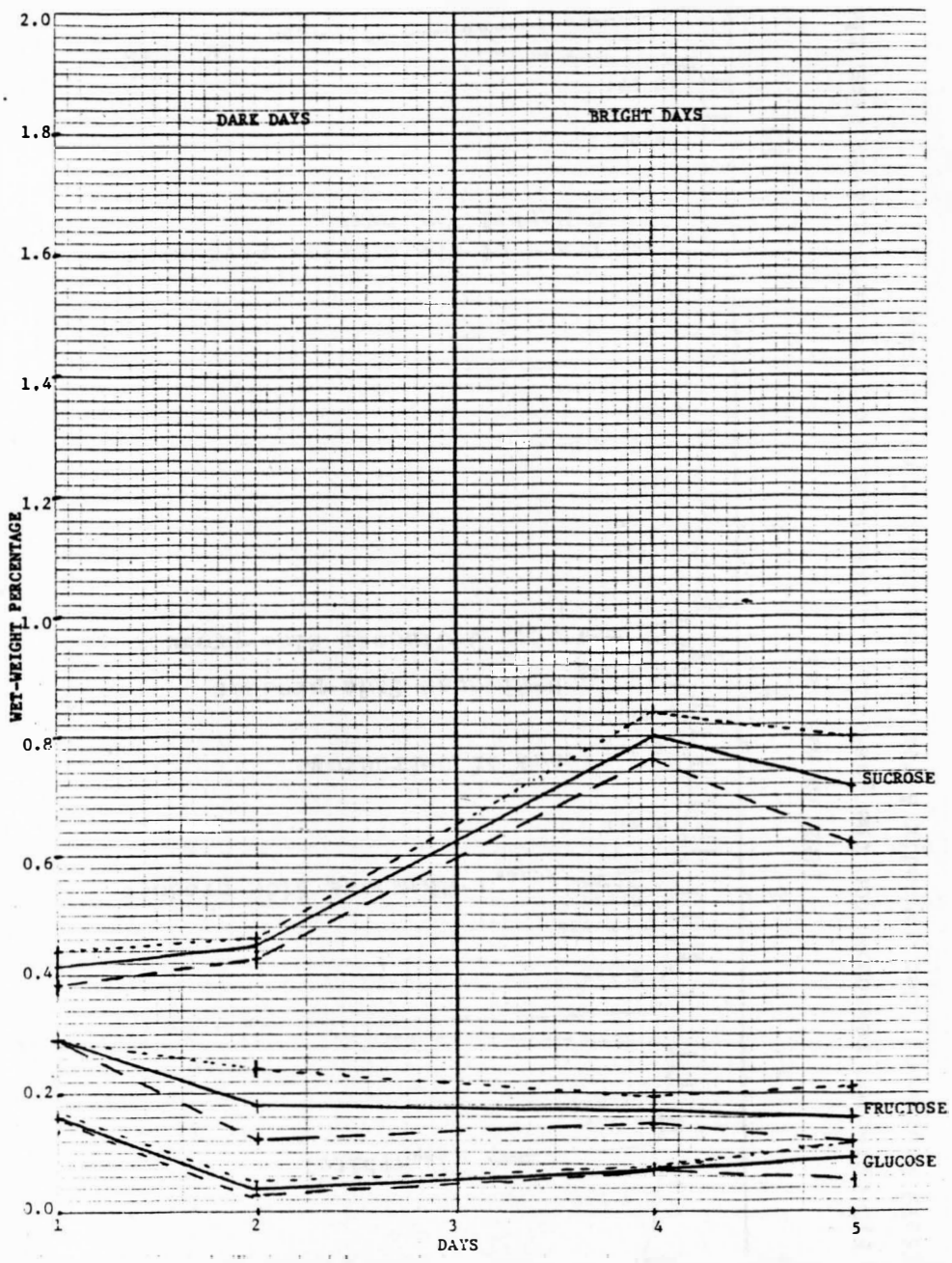


Figure 11. Wet-weight sugar percentages in the stem tissues of Canada thistle as a function of time (in days). The dotted and dashed lines refer to specific growing areas, the solid line to the average value from the two areas.

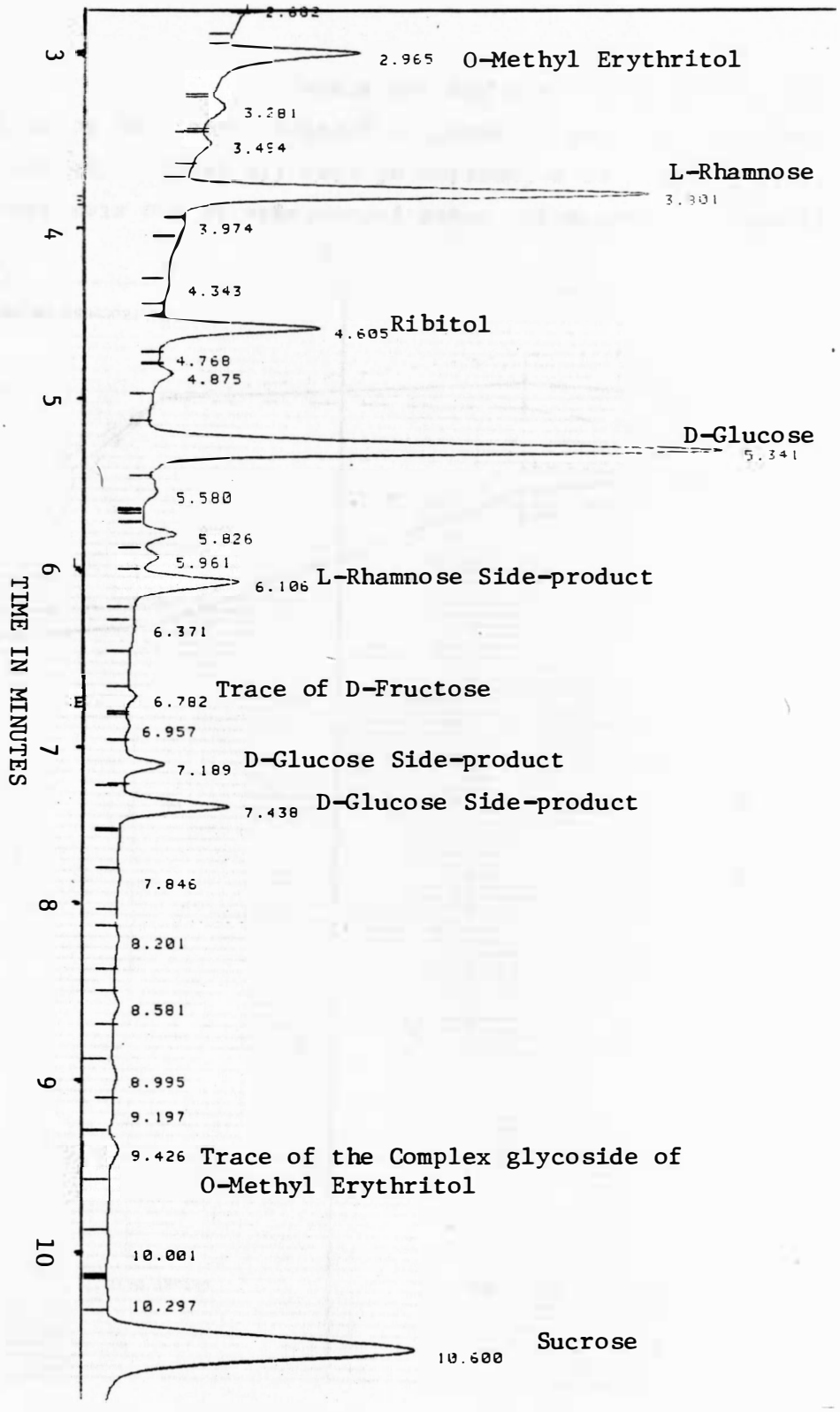


Figure 12. The FID-detector response for the gas-liquid chromatogram of the derivatives from the stem-tissue of field bindweed. See Appendix A for chromatographic conditions.

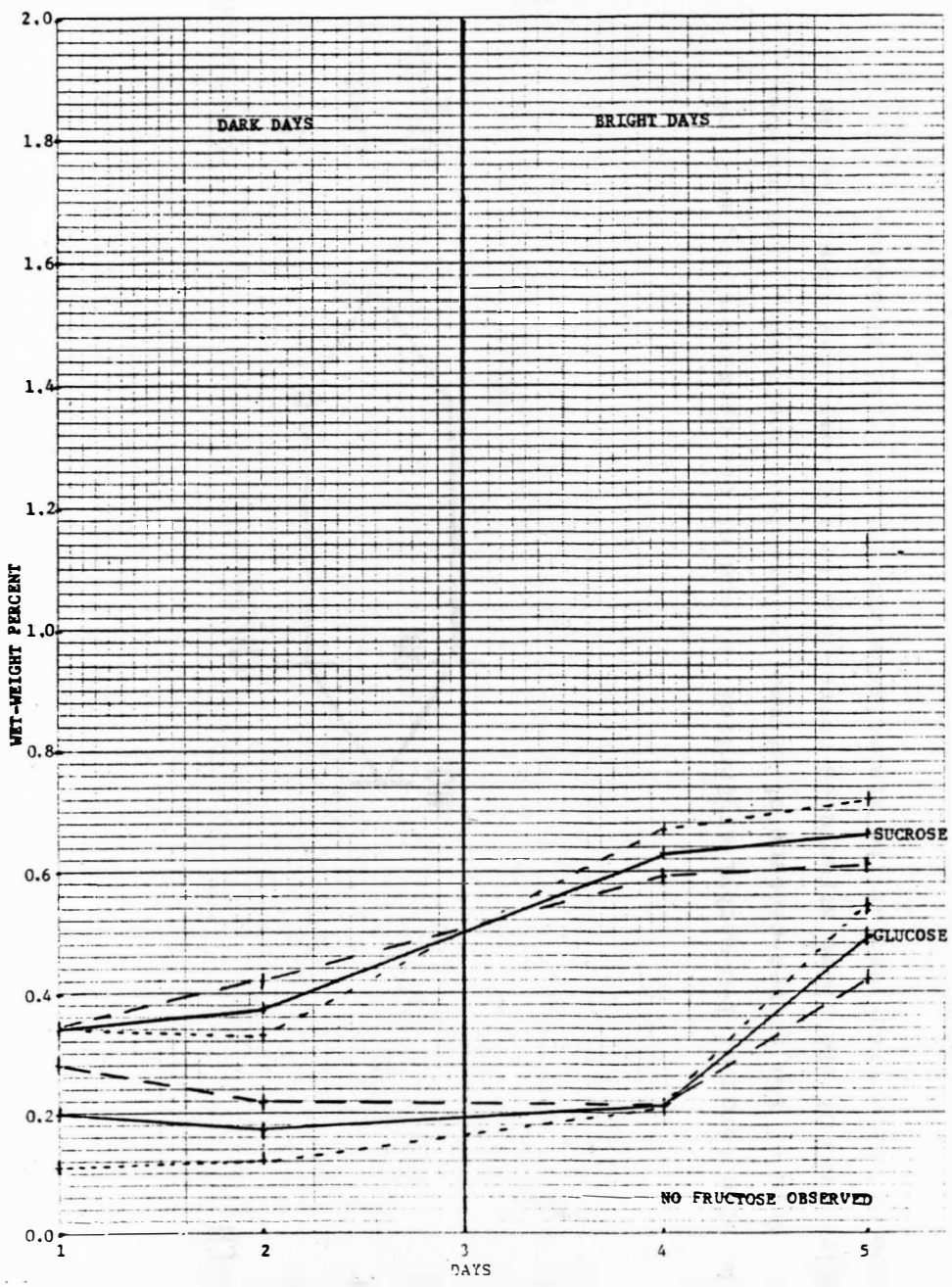


Figure 13. Wet-weight sugar percentages in the stem tissues of Field bindweed as a function of time (in days). The dotted and dashed lines refer to specific growing areas, the solid line to the average value from the two areas.

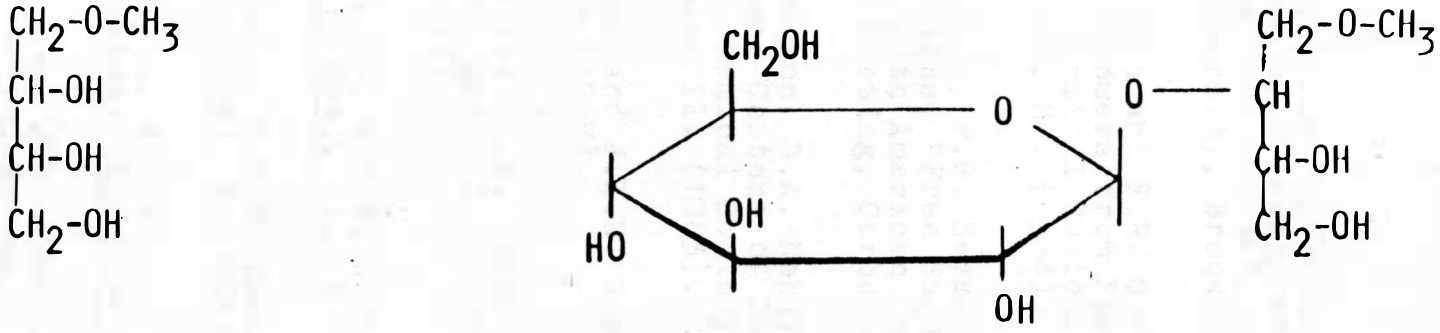


Figure 14. The structures of 1-O-methyl erythritol (a) and of the corresponding complex glycoside (b). None of the stereo-centers of asymmetry have been confirmed. The per-O-acetylated derivatives of the above structures correspond to the compounds identified in the chromatogram of the derivatives of the stem-tissue extract of field bindweed.

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